



*UNRAVELING THE MECHANISM OF THE
LIGHTSTRUCK FLAVOR OF BEER*

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Conventions

For the sake of clarity, compounds are labelled per chapter. The first digit indicates the chapter number, the second digit refers to the order of appearance in the chapter. Labels a-c are for the isomers/homologs of the hop-derived substances (a: R = i-butyl; b: R = i-propyl; c: R = sec-butyl). References are also arranged per chapter.

Abbreviations

| | |
|-------|--|
| A/E | absorptive/emissive |
| amu | atomic mass units |
| API | atmospheric pressure ionization |
| CID | collision induced dissociation |
| CIDEP | chemically induced dynamic electron polarization |
| CW | continuous wave |
| D | zero field splitting parameter |
| DAD | diode array detector |
| DCHA | dicyclohexylamine |
| DNA | deoxyribonucleic acids |
| E/A | emissive/absorptive |
| EDTA | ethylenediaminetetraacetic acid |
| EPR | electron paramagnetic resonance |
| ESI | electrospray ionization |
| ESR | electron spin resonance |
| FAD | flavin adenine dinucleotide |
| FMN | flavin mononucleotide |
| FMW | field modulation width |
| FPD | flame photometric detection |
| GC | gas chromatography |
| HPLC | high performance (or pressure) liquid chromatography |
| ISC | intersystem crossing |
| ISO | isohumulones |
| LF | lumiflavin |
| LSF | lightstruck flavor |
| MBT | 3-methylbut-2-enyl-1-thiol |
| MS | mass spectroscopy |
| MW | molecular weight |
| NMR | nuclear magnetic resonance |
| OMA | optical multichannel analyzer |
| PM | photomultiplier |
| RF | riboflavin |

| | |
|--------|---|
| RP | reversed phase |
| RPM | radical pair mechanism |
| RTPM | radical triplet pair mechanism |
| S/N | signal to noise ratio |
| SCRP | spin correlated radical pair mechanism |
| SSEPR | steady-state electron paramagnetic resonance |
| TEMPO | 2,2,6,6-tetramethylpiperidine- <i>N</i> -oxyl |
| TM | triplet mechanism |
| t_R | retention time |
| TREPR | time-resolved electron paramagnetic resonance |
| UV | ultraviolet |
| UV/Vis | ultraviolet/visible |
| YAG | yttrium aluminium garnet |

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BACKGROUND INFORMATION AND LITERATURE REVIEW

CHAPTER 1

BEER AND LIGHTSTRUCK FLAVOR: HISTORICAL PERSPECTIVE AND GENERALITIES

Light exposure has a detrimental effect on the quality of a variety of drinks. Next to milk and fruit juices, all fermented non-distilled beverages suffer from light-induced development of off-flavors and deterioration of the product. Such beverages contain vitamin B2 (riboflavin), a yellow-colored compound known for its versatile (photo)chemistry, as a key component in the photodegradation of ingredients. Irradiation of milk leads to a decrease in vitamin B2 (riboflavin) content, along with color changes¹ and production of methional.² The appearance of off-flavors in champagne and wine, often described as ‘cooked cabbage’ and ‘onion-garlic’, is linked to photogeneration of thiols (methylthiol and dihydrogen sulfide) and dimethyldisulfide.³ Beer, upon exposure to light, stands out due to the development of a very specific, obnoxious skunky off-flavor. The compound responsible for the ‘skunkiness’ was identified as 3-methylbut-2-ene-1-thiol.⁴

1.1 LIGHTSTRUCK FLAVOR IN BEER: AN AGE OF INVESTIGATIONS

1.1.1 Early reports

Lintner highlighted the lightstruck flavor (LSF) of beer already in 1875.⁵ This finding was the onset of more than an age of investigations. In 1908, Brand showed that sunlight had no effect on the flavor of hopped wort and concluded that some of the compounds involved in the development of the LSF are produced during fermentation.⁶ Furthermore, dark-brown colored bottles proved most effective in inhibiting the formation of the LSF. In 1934, De Clerck indicated that development of a lightstruck character coincided with a decrease in redox potential.⁷ Gray *et al.* confirmed this finding in 1941 by showing that copper ions, as well as molecular oxygen reduced the tendency of beer to develop the LSF.⁸ They were also the first to show that low-molecular-weight thiols were present in lightstruck beer. Jacobssen and Högberg (1947) used thiol-binding agents to highlight the importance of thiols.⁹

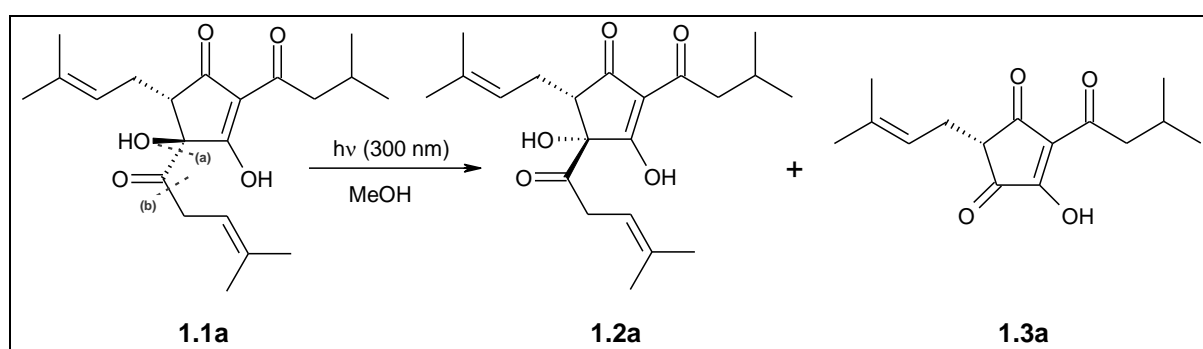
1.1.2 Kuroiwa formalism

In the early sixties, Kuroiwa *et al.* established much of the basic chemistry underlying the formation of LSF in beer. First, they found that unhopped beer did not produce the typical skunky flavor. Addition of the hop-derived beer-bittering isohumulones reinstalled the potential for formation of LSF.¹⁰ Further studies using model systems showed that LSF was produced in a non-enzymic light-induced reaction involving a flavin (as a sensitizer), isohumulones, and a suitable sulfur-containing compound.¹¹⁻¹³ The typical skunky flavor was attributed to the formation of 3-methylbut-2-ene-1-thiol (MBT). The effective wavelength range for formation of the LSF deduced from sensory analysis was found to be 350-500 nm.¹⁴ On the other hand, oxygen and other oxidizing agents such as hydrogen peroxide and potassium permanganate were found to suppress formation of MBT in model systems.¹⁵ Independently, Obata *et al.* observed the typical LSF of beer on addition of trace amounts of synthetic MBT to a fermented solution of sucrose.¹⁶ In 1978, Gunst and Verzele confirmed unambiguously that the content of MBT increased on illumination of beer. They used GC analysis with flame photometric detection after concentrating the sample on Porapak Q,⁴ while MBT was identified by the retention time, by co-chromatography with synthetic MBT and by mass spectroscopy. However, LSF does not arise solely by formation of MBT. Although MBT is governing the overall off-flavor due to its specific flavor and low threshold, formation of other sulfur-containing compounds on exposure of beer to light including dihydrogen sulfide and methylthiol, as found by Kattein *et al.*, suggests a contribution to the overall off-flavor.¹⁷

1.1.3 Further mechanistic investigations

In 1987, Blondeel *et al.* reported that isohumulones showed photoreactivity when irradiated in the lower UV-wavelength range.¹⁸ While irradiation of *trans*-isohumulone (**1.1a**) in deaerated methanol at 254 nm led to a very complex mixture containing no major constituents, only two major photoproducts were found on irradiation at 300 nm (**Scheme 1.1**), which were identified as *cis*-isohumulone (**1.2a**) and dehydrohumulinic acid (**1.3a**). Their formation could be rationalized in a straightforward manner by light-induced α -cleavage of the α -hydroxyketone group in the side chain at C-4, the so-called Norrish Type I α -cleavage. Two fragmentation pathways can be envisaged: either α -cleavage of the α -

hydroxyketone (**a**) or α -cleavage in the acyclic β,γ -enone part (**b**) (**Scheme 1.1**). In both instances, a stabilized radical pair is formed. Ready recombination accounts for the low quantum yield, while *cis*-isohumulone results from epimerization of the ketyl radical center. Dehydrohumulinic acid is formed from the ketyl radical, derived either directly via route (**a**) or after decarbonylation of the acyl radical from route (**b**) by α -hydrogen elimination. It was suggested that Norrish Type I α -cleavage arose from the (n,π^*) -triplet manifold. Formation of dehydrohumulinic acid provided the first direct proof for photodegradation of *trans*-isohumulone (and, also, of isohumulones in general) with concurrent release of a precursor of MBT. It is, therefore, the key route to the development of LSF on UV irradiation. No other hop components seem to be involved in the process.



Scheme 1.1

Major photoreaction products formed on irradiation (300 nm) of *trans*-isohumulone.

The Kirin research group developed an analytical technique based on a purge-and-trap method, in combination with GC-analysis using flame photometric detection for quantification of MBT formed on exposure of beer to light.¹⁹ With different bottles, but with an identical beer, the amount of MBT formed increased proportionally to the ability of the bottle to transmit light between 350 nm and 500 nm (**Figure 1.1**). When comparing different beer brands in identical bottles, significant differences were found in the total amount of MBT after exposure to light. In order to understand the influence of various beer components on formation of MBT, four model solutions were prepared: A: isohumulones, cystine, and riboflavin; B: isohumulones, a sulfur-containing protein fraction, and riboflavin; C: isohumulones, dihydrogen sulfide, ascorbic acid, and riboflavin; D: isohumulones, dihydrogen sulfide, and riboflavin. All solutions were exposed to sunlight and the concentrations of MBT were determined. In solutions A and B, MBT was not detected in the absence of riboflavin, while the concentration of MBT increased with the amount riboflavin added. In contrast, MBT was detected in solution C only when the concentration of ascorbic

acid reached 50 mg/L. In solution D, MBT was absent. The results of these experiments clearly show that photochemical sensitization by riboflavin was indispensable for formation of MBT from isohumulones and from sulfur-containing amino acids or proteins, and that a high level of ascorbic acid was necessary for formation of MBT from isohumulones and dihydrogen sulfide.

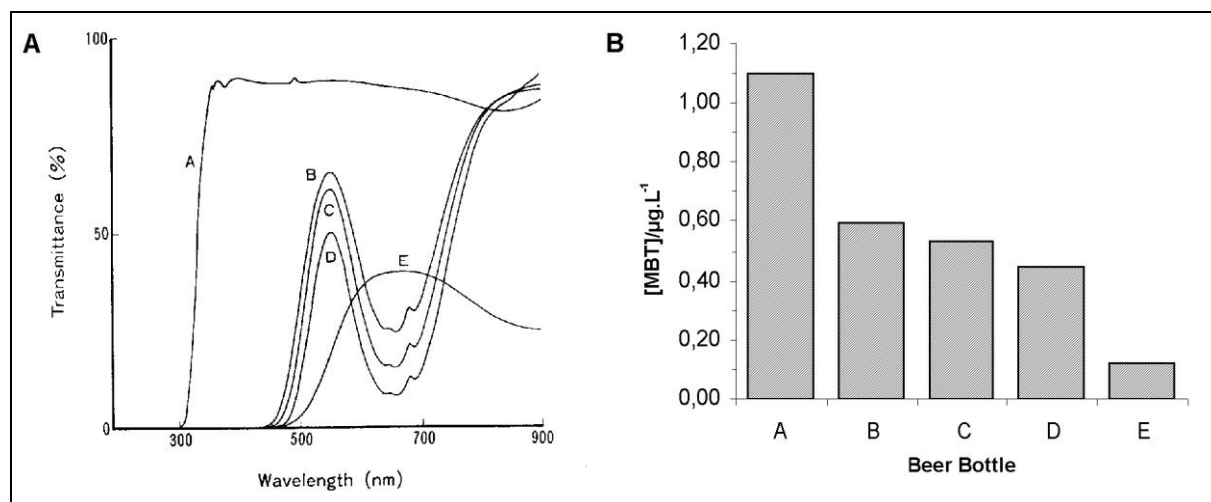


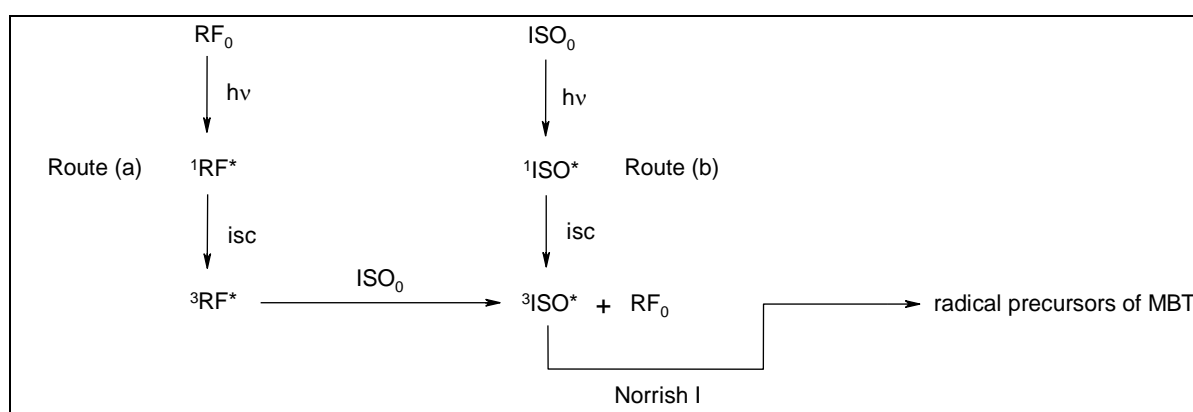
Figure 1.1

A: Light transmittance patterns of various beer bottles.

B: Formation of 3-methylbut-2-ene-1-thiol (MBT) in a lager beer exposed to sunlight in different bottles: A: clear bottle; B, C, D: green bottles; E: brown bottle.

To further clarify the contribution of beer components to the formation of MBT, compounds implicated in the development of LSF were added to a beer in green-colored bottles and the beers were exposed to sunlight. Formation of MBT was greatly accelerated by addition of both isohumulones and riboflavin. Sulfur-containing amino acids and ascorbic acid also led to an increase in the contents of MBT, dihydrogen sulfide had no effect, and sulfite suppressed its formation. Furthermore, intensifying the beer color gradually eliminated formation of MBT. From these results, Sakuma *et al.* concluded that the main route to MBT on exposure to sunlight involves decomposition of isohumulones decompose to a 3-methylbut-2-enyl radical, while sulfur-containing amino acids and proteins are a source of thiol radicals in riboflavin-photosensitized reactions. Both radicals then combine to form MBT. Also, the amount of riboflavin in beer is an important factor resulting in differences in beer brands regarding formation of LSF. They proposed that, if riboflavin could be removed from beer, a light-stable beer might be obtained.

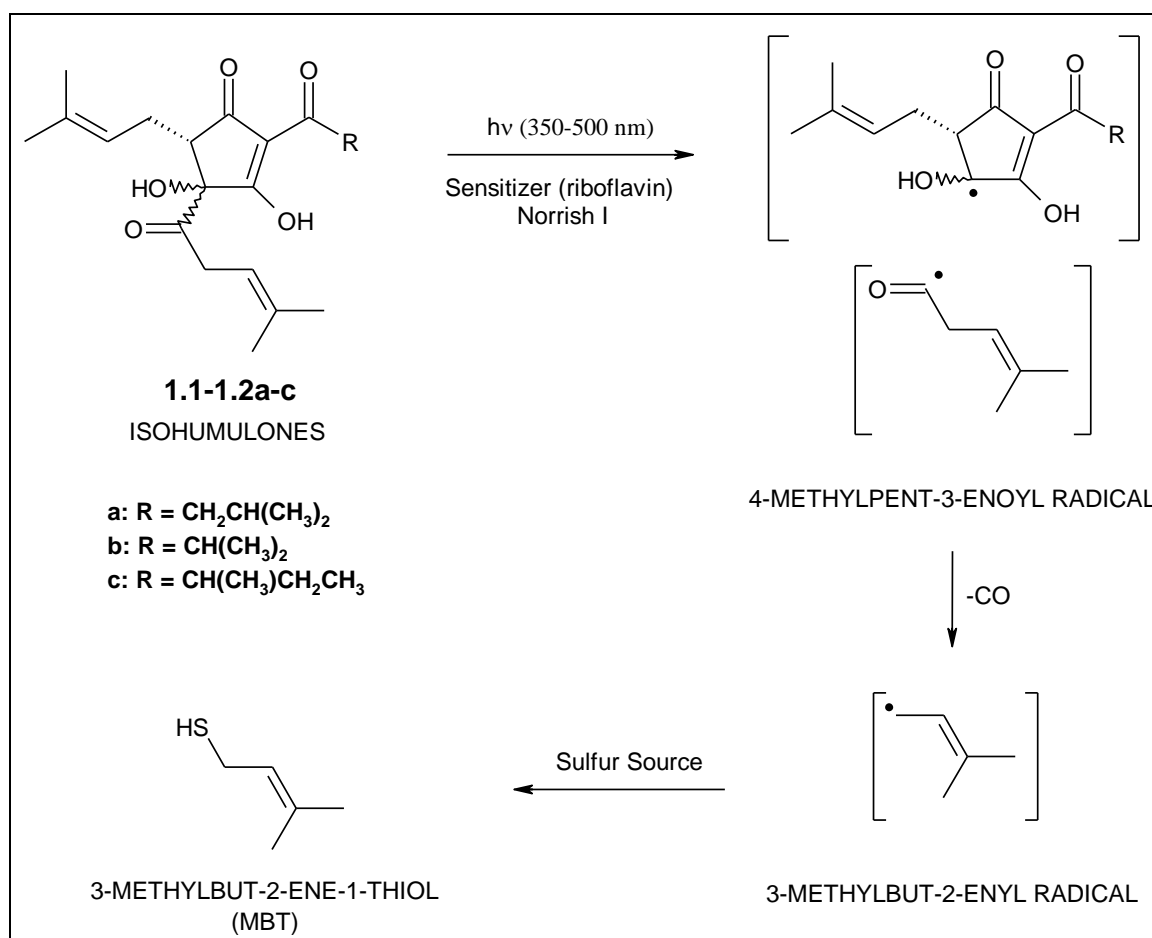
A formal mechanism for formation of LSF in beer was commonly accepted (**Schemes 1.2, Scheme 1.3**). According to route (a), riboflavin is excited to the singlet state by light in the wavelength range of 350-500 nm. The excited singlet state gives rise to an excited triplet state via intersystem crossing (isc). Subsequent triplet energy transfer to isohumulones in the ground state leads to triplet-excited isohumulones. From the excited triplet state (also formed on direct irradiation, route (b)), Norrish Type I α -cleavage of the α -hydroxyketone group furnishes a 4-methylpent-3-enoyl radical, which undergoes decarbonylation to a 3-methylbut-2-enyl radical. Trapping of this stabilized allyl radical by a suitable sulfur source (e.g. cysteine) leads to formation of 3-methylbut-2-ene-1-thiol (MBT).



Scheme 1.2

Formal mechanism for direct and photosensitized (RF: riboflavin) irradiation of isohumulones (ISO) furnishing radical precursors for formation of 3-methylbut-2-ene-1-thiol (MBT).

This proposal still holds on, although it was proven incorrect by Hastings *et al.* in 1992.²⁰ From the absorption and phosphorescence spectra of both riboflavin and *trans*-isohumulone, they were able to conclude that the wavelengths separating the excited triplet state from the ground state of riboflavin and *trans*-isohumulone corresponded to ca. 560 nm and 395 nm, respectively. These wavelengths are associated to triplet energies of 210 kJ/mol for riboflavin and 300 kJ/mol for *trans*-isohumulone. Direct uphill triplet energy transfer by 90 kJ/mol can be ruled out on the basis of thermodynamic considerations.



Scheme 1.3

Formal mechanism for light-induced formation of 3-methylbut-2-ene-1-thiol (MBT) according to Kuriowa *et al.*¹²

With the help of a panel of 16 experienced beer tasters, Irwin *et al.*²¹ determined the flavor threshold of MBT in beer to be in the range of 4.4-35 ng.L⁻¹ (varying from person to person), clearly establishing MBT as one of the organoleptically most potent substances known. Furthermore, they investigated in model systems whether the rate of development of LSF was limited by the concentrations of cysteine or riboflavin. While keeping the concentrations of both isohumulones and riboflavin constant, LSF was quite intense even for the lowest concentrations of cysteine, suggesting that cysteine is not involved in the rate-determining step of the reaction (**Figure 1.2A**). On the other hand, the intensity of LSF steadily increased as a function of the concentration of riboflavin, which indicates the involvement of riboflavin in the rate-determining step (**Figure 1.2B**). Therefore, lowering the concentration of riboflavin in beer would be more effective in reducing the sensitivity of beer to light than would be lowering the concentration of cysteine.

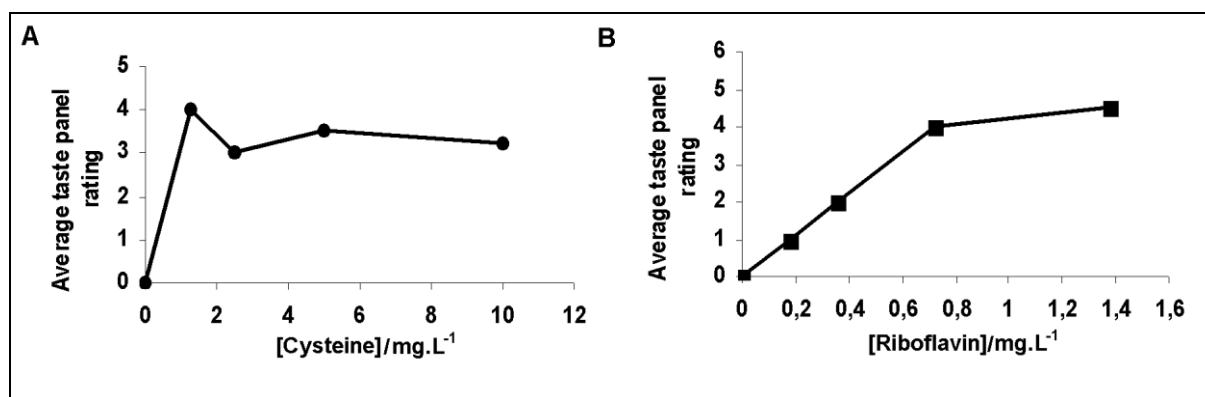


Figure 1.2

A: Effect of the concentration of cysteine on the intensity of the lightstruck flavor (riboflavin: 1.4 mg.L⁻¹; isohumulones: 10 mg.L⁻¹).

B: Effect of the concentration riboflavin on the intensity of the lightstruck flavor (cysteine: 10 mg.L⁻¹; isohumulones 10 mg.L⁻¹).

1.1.4 Quantitative analysis of 3-methylbut-2-ene-1-thiol

While the analyses of 3-methylbut-2-ene-1-thiol (MBT) in beer by Sakuma *et al.* were rather qualitative and at levels far above the sensory threshold, an accurate and sensitive quantitative gas chromatographic method was developed by Goldstein *et al.* in 1993.²² The importance relates to the fact that sensory panelists are dubious in distinguishing progressively higher concentrations of MBT. The sensory threshold of MBT was determined to be 0.2-0.3 ng.L⁻¹ in water²³ and 1.25-2.50 ng.L⁻¹ in an American lager beer. Using a sample volume of 4.26 L and a thiol-specific trap consisting of Hg (II) cyanide-containing glass wool, they arrived at limits of detection as low as 2 ng.L⁻¹ for flame ionization detection and flame photometric detection, 1 ng.L⁻¹ for chemiluminescence detection, and less than 1 ng.L⁻¹ for mass spectroscopic detection. Interestingly, concentrations of 1-5 ng.L⁻¹ were found in beers containing isohumulones prior to illumination, probably originating from a thermal reaction after kettle hopping. In 1997, Hughes *et al.* developed a purge-and-trap method for quantification of MBT using a Tenax trap and gas chromatography in combination with a Sievers chemiluminescence detector.²⁴ The limit of detection was ca. 4 ng.L⁻¹. Furthermore, concentrations of other sulfur volatiles were monitored as a function of irradiation time. It was apparent that, after prolonged illumination, beers developed methylthiol and dihydrogen sulfide to different extents varying within different brands. Recently, Masuda *et al.* optimized the method using Tenax GR and slightly different purge conditions, followed by GC-MS.²⁵ Using mass spectroscopic detection, they were able to lower the detection limit to 0.2 ng.L⁻¹. The flavor threshold of MBT in Japanese beers was found to be ca. 2 ng.L⁻¹.

1.2 ROLE OF HOPS

1.2.1 Botanical description

The hop plant belongs to the family of the Cannabaceae,²⁶ consisting of two genera: *Humulus* and *Cannabis*. *Humulus* exists as two species, *Humulus lupulus* L., which has a high economical value because of its use in beer brewing, and *Humulus japonicus* Sieb. & Zucc., which is only grown as an ornamental plant. The hop plant is a fast growing vine (up to 6-7 m per year) with unisexual male and female flowers growing on separate plants. Only the female flowers are important for commercial purposes, while they contain all compounds of interest for brewing. Day-length requirements restrict cultivation of hops to latitudes between 35° and 55° in both hemispheres. Further requirements such as fertile soil, relatively high summer temperatures, and access to water via rain, ground water or irrigation are met in the larger hop growing areas situated in Europe (Germany, Czech Republic, England), the United States of America (mainly in the States of Washington and Oregon), Australia, and New-Zealand. In the northern hemisphere, the female flowers start to grow during July and the hop cones can be harvested from the end of August to the end of September, depending on hop variety and weather conditions.

1.2.2 Chemical composition of the hop cones

The female hop flowers contain, next to the primary metabolites, hundreds of secondary metabolites^a comprising many different groups of organic compounds. Of particular interest are:

- a) hop resins, containing the hop acids;
- b) hop essential oil;
- c) hop polyphenols.

These three classes are not only important because of their profound impact on various beer characteristics, but they are also useful as significant biochemical markers in varietal

^a Secondary metabolites are distinguished from primary metabolites by the following criteria: they have a restricted distribution being found mostly in plants and micro-organisms, and are often characteristic of individual genera, species, or strains; they are formed along specialized pathways from primary metabolites. Secondary metabolites are non-essential to life, although they are important to the organism that produces them in correlation to environmental factors or stress.

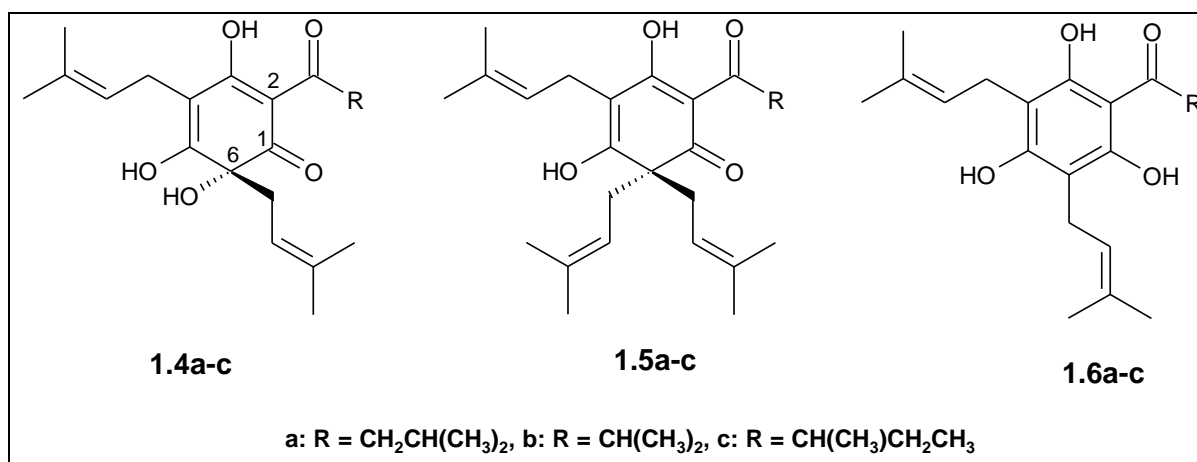
characterization of different hop cultivars.²⁷ The major components of dried hop cones are listed in **Table 1.1**.

Table 1.1
Average composition of dried female hop cones.

| Components | Percentage (w/w) |
|------------------------|------------------|
| α -Acids | 2-19 |
| β -Acids | 2-11 |
| Essential Oil | 0.5-3.0 (v/w) |
| Polyphenols | 3-6 |
| Monosaccharides | 2-3 |
| Amino acids | 0.1 |
| Proteins | 15 |
| Lipids and fatty acids | 1-5 |
| Pectins | 2 |
| Ashes – salts | 5-10 |
| Cellulose – lignins | 40-50 |
| Water | 8-12 |

1.2.2.1 Hop acids

Hop acids are the most important constituents of the so-called hop resins. They have, until now, not been found in any other plant, thus showing a high chemotaxonomical value. Remarkably, hop acids can constitute up to ca. 30% of the total weight of dried female hop cones. Generally, concentrations of secondary metabolites are quite low, while they can be toxic for the plant itself. Apparently, the hop plant has circumvented this situation by secreting the hop resins in extra-cellular organs, the so-called lupulin glands. Hop acids comprise two related classes, the α -acids (α because they were discovered first) and the β -acids.²⁸ These weak organic acids are only slightly soluble in water and have a faint bitter taste. The denotation ‘*hop bitter acids*’ refers to the fact that the α -acids function as precursors for the hop-derived bittering principles in beer, the *iso*- α -acids. α -Acids or *humulones* (**1.4a-c**) (named after the major component in the mixture of α -acids) consist of three major analogs (two isomers and one homolog), humulone (**1.4a**), cohumulone (**1.4b**), and adhumulone (**1.4c**), which differ only in the nature of the side chain at C-2 due to biogenetic incorporation of different hydrophobic amino acid residues, derived from leucine, valine, and isoleucine, respectively. Similarly, β -acids or *lupulones* (**1.5a-c**) also consist of three major analogs, namely lupulone (**1.5a**), colupulone (**1.5b**), and adlupulone (**1.5c**).



Scheme 1.4

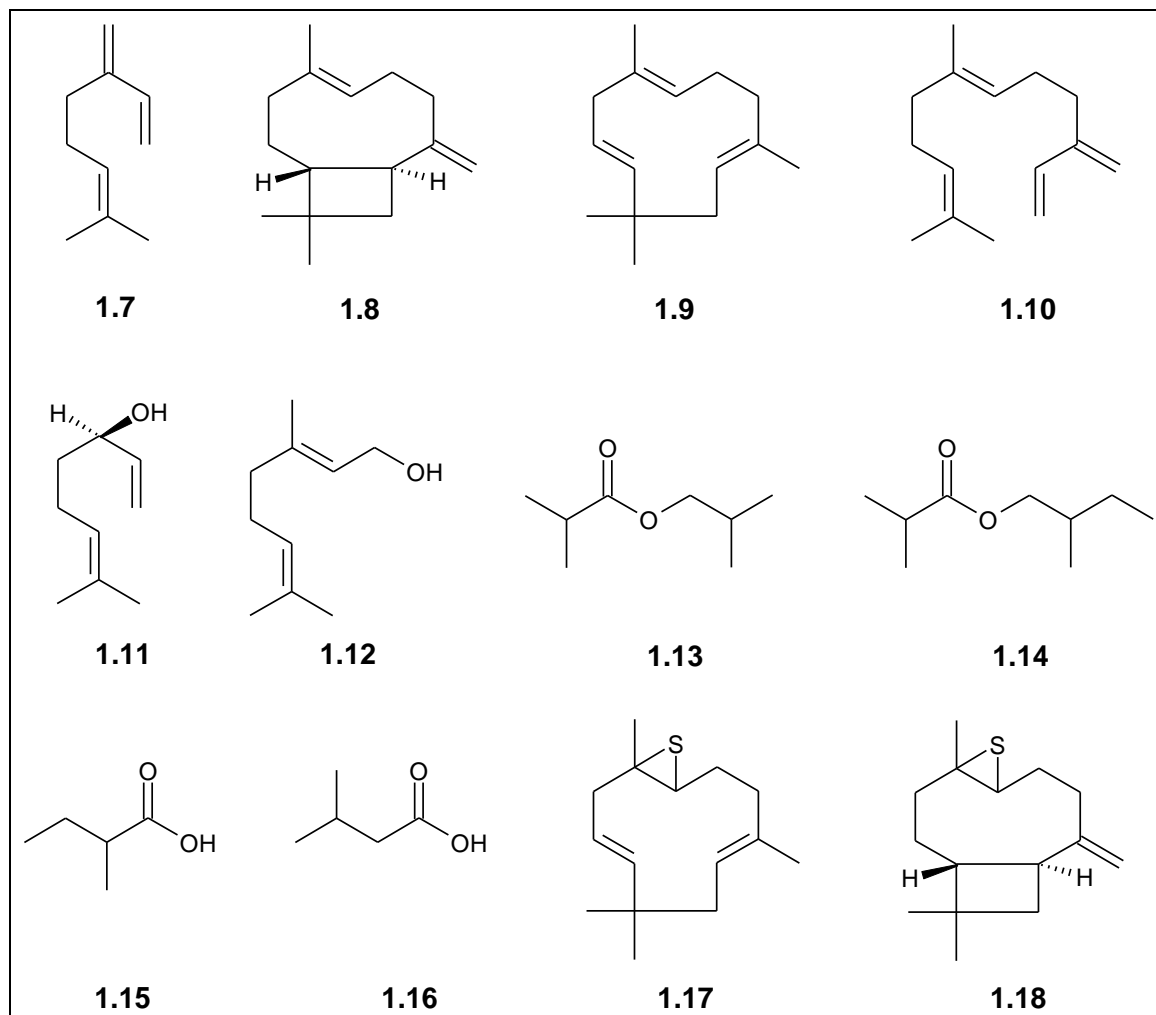
Structures of humulones, lupulones, and 6-deoxy- α -acids.

Substitution of a prenyl side chain in lupulones for the tertiary hydroxyl group at C-6 in humulones has a profound effect on the chemical properties. While both series of hop acids are closely related through their common biochemical precursors, known as 6-deoxy- α -acids (**1.6a-c**), they exhibit a completely different behavior during wort boiling with hops. At the pH of wort, the solubility of humulone is ca. 40 mg.L⁻¹ at 25°C and 60 mg.L⁻¹ at 100°C, while the solubility of lupulone is only ca. 1 mg.L⁻¹ at 25°C and 9 mg.L⁻¹ at 100°C. Any additional material dissolved at the boiling point will precipitate on cooling. However, during wort boiling, the humulones (pK_a~5) are isomerized to the isohumulones (pK_a~3) which are much more soluble in the aqueous matrix.²⁸ In contrast, lupulones cannot undergo the facile isomerization reaction, while they lack the tertiary alcohol function as part of the acyloin group required for the acyloin rearrangement. Instead, lupulones are susceptible to extensive oxidative degradation, mainly involving the double bonds of the prenyl side chains.

1.2.2.2 Hop essential oil

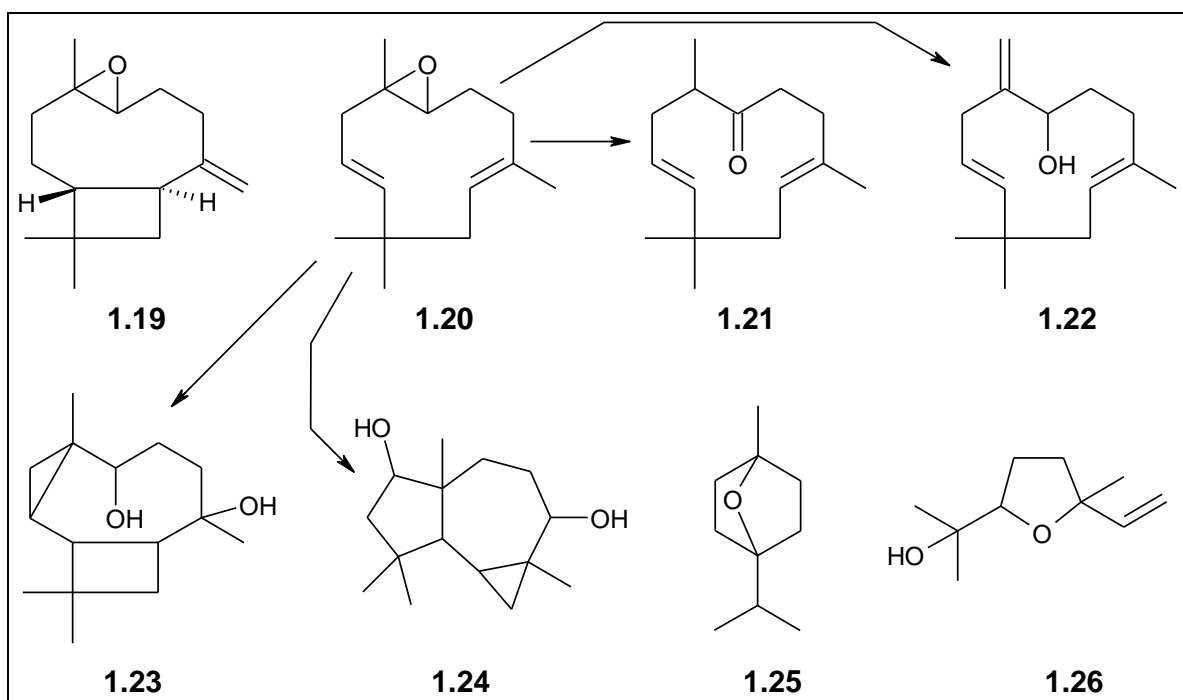
Hop essential oil represents a small, volatile fraction of hops (0.5-3.0% v/w), in which ca. 300 compounds have been positively or tentatively identified.^{29,30} The complex mixture can readily be separated into a hydrocarbon fraction (40-80% of the total oil) and an oxygenated fraction.³¹ Three or four major compounds are prominent in the hydrocarbon fraction, the monoterpene β -myrcene (**1.7**) and the sesquiterpenes β -caryophyllene (**1.8**), α -humulene (**1.9**), and, sometimes, β -farnesene (**1.10**).³² Most aroma hops contain relatively high levels of α -humulene and low levels of β -myrcene. α -Humulene is being held responsible, at least in

part and in combination with a number of other constituents of the essential oil, for the pleasant smell of hops. β -Myrcene and the sesquiterpenes are notoriously reactive, and, thus, a great variety of oxidized terpenes can be found in the oxygenated fraction.



Scheme 1.5
Important constituents of the essential oil of hops.

Linalool (**1.11**), the major monoterpene alcohol in hops, and geraniol (**1.12**), are examples of oxygenated terpenes that produce floral scents. Other important oxygenated products include a variety of sesquiterpenoid alcohols and epoxides. Furthermore, many esters such as 2-methylpropyl isobutyrate (**1.13**) and 2-methylbutyl isobutyrate (**1.14**) contribute to the fruity aroma of hops, whereas fatty acids, mainly 2-methylbutyric acid (**1.15**) and 3-methylbutyric acid (**1.16**), give the cheesy aroma of old hops. Sulfur compounds include thiols, sulfides, polysulfides, thioesters, thiophenes, and episulfides such as 1,2-epithio- α -humulene (**1.17**) and epithiocaryophyllene (**1.18**). In addition, few aliphatic aldehydes and methyl ketones have been reported to occur in hop oil.



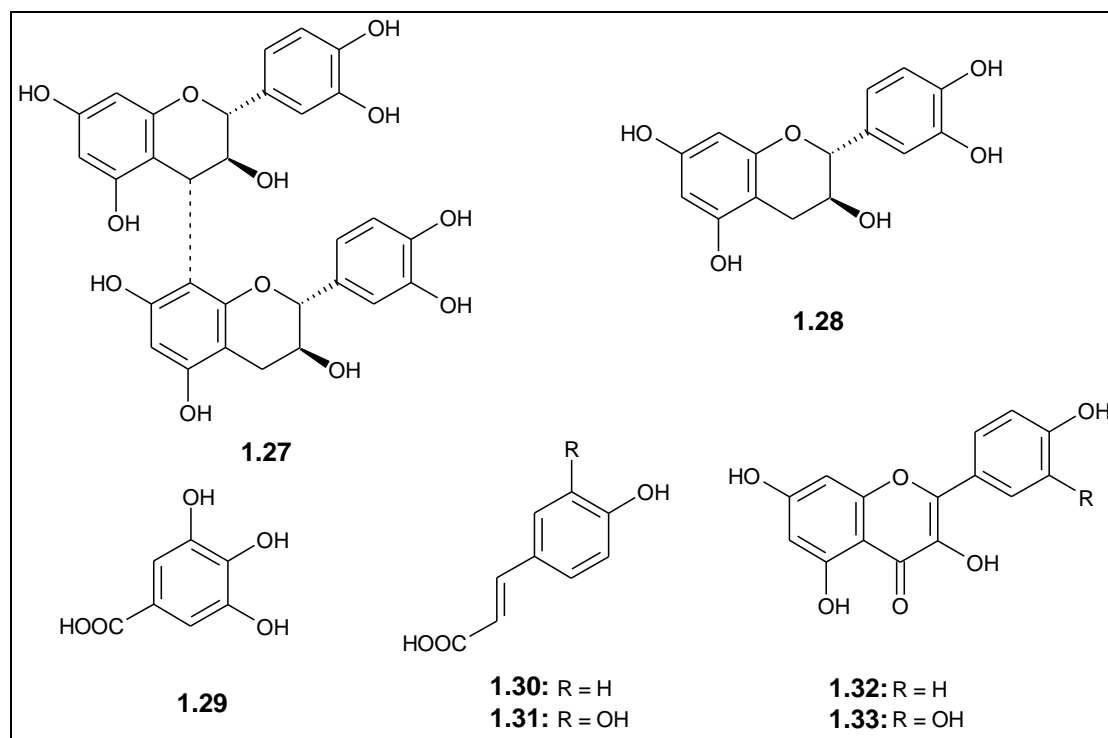
Scheme 1.6
Oxygenated hop-derived flavor compounds in beer.

It is important to notice that many constituents of the hop essential oil are lost or transformed during the brewing process. It has been advocated that the hoppy character of beer is mainly due to oxygenated terpenoids. Sesquiterpene hydrocarbons are very susceptible to auto-oxidation, whereby mainly epoxides are formed, such as β -caryophyllene epoxide (**1.19**) and α -humulene-1,2-epoxide (**1.20**).³³ The reactivity of epoxides induces formation of a variety of oxidized derivatives. For example, reactions of α -humulene-1,2-epoxide in boiling wort lead to a complex mixture, including humuladienone (**1.21**),³⁴ β -humulene-1-ol (**1.22**)³⁵ and tricyclohumuladiols (**1.23-1.24**).³⁶ Also monoterpene cyclic ethers such as 1,4-cineole (**1.25**) and linalool oxide (**1.26**) are important flavor compounds.

1.2.2.3 Hop polyphenols

The complex nature of hop polyphenolic extracts is readily shown by reversed-phase HPLC analysis.^{37,38} Hop polyphenols, constituting 3-6% w/w of the total weight of dried female hop cones, can roughly be divided in four major subclasses. The first eluting fraction consists merely of proanthocyanidins, a mixture of monomers, dimers (e.g. procyanidine B3 (**1.27**)), and oligomers, composed of catechin (**1.28**) units. Next elutes a small fraction containing phenolic acids including both hydroxybenzoic acids (e.g. gallic acid (**1.29**)) and

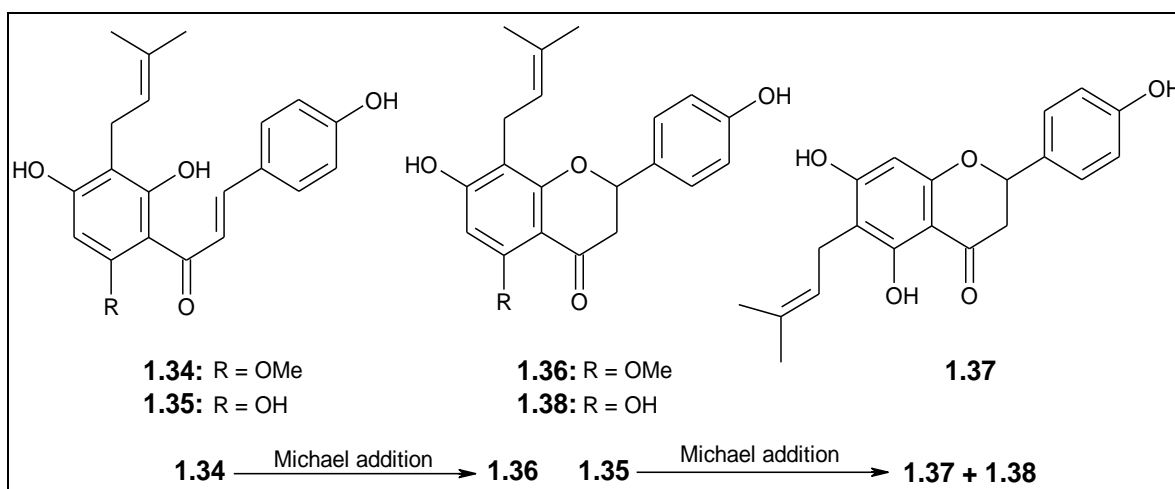
hydroxycinnamic acids (e.g. coumaric acid (**1.30**) and caffeic acid (**1.31**)). The largest fraction of polyphenols in hops consists of the flavonols kaempferol (**1.32**) and quercetin (**1.33**), which prevail mostly as glycosides.



Scheme 1.7
Important polyphenolic constituents of hops.

The chalcones xanthohumol (**1.34**) and desmethylxanthohumol (**1.35**) are the most important late eluting polyphenols. Their corresponding flavanones, isoxanthohumol (**1.36**), and 6-prenylnaringenin (**1.37**) and 8-prenylnaringenin (**1.38**), respectively, were detected and quantified in polyphenolic hop extracts and in beer.³⁹⁻⁴² Furthermore, 8-prenylnaringenin was shown to be the most potent phytoestrogen known to date.^{43,44}

With 20-30% of polyphenols in wort being derived from hops,⁴⁵ it can be expected that the overall beer quality is influenced to some extent by hop polyphenols. Whereas some indications point to a positive contribution of polyphenols to beer mouthfeel, others associate a harsh astringent flavor to these polyphenols. Furthermore, hop polyphenols contribute partly to the color of beer. Polyphenols also act as natural antioxidants, thereby contributing to a large extent to the reductive power of wort and, at a later stage, to prevention of staling.



Scheme 1.8

Prenylated chalcones and flavanones in hops.

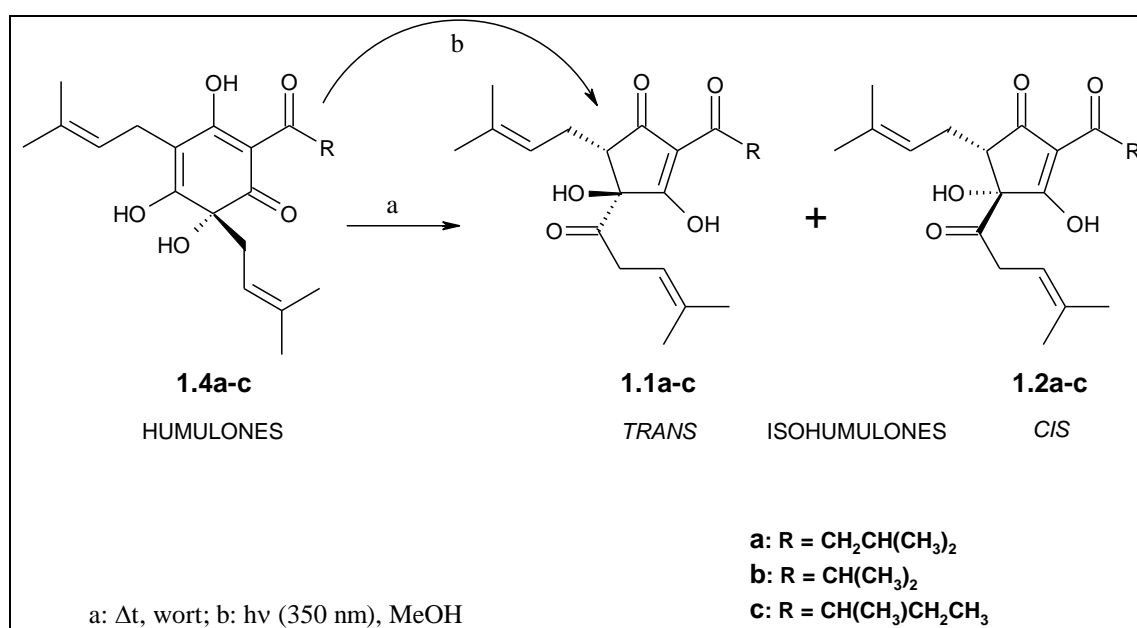
Higher-molecular-weight polyphenols (500-3000 amu) such as dimeric and oligomeric proanthocyanidins are also known as tannoids, while they tend to form aggregates with proteins, which then precipitate. Thus, these polyphenols play a significant role in the colloidal stability of beer.⁴⁶ A first manifestation of the loss of colloidal stability is observed as chill haze, which is caused by a reversible association of higher-molecular-weight polyphenols and proteinaceous material.⁴⁷ Chill haze redissolves when warmed up. The tendency to form chill haze increases progressively over time. Initially, simple monomeric and dimeric proanthocyanidins form hydrogen bonds with soluble proteins without causing turbidity. Oxidation and polymerization of simple flavanols during storage lead to formation of flavanol-type oligomers that are able to aggregate with soluble proteins, some of which can form chill haze on cooling. As the polyphenols continue to oxidize, larger complexes are formed and supramolecular bonds are partly substituted for covalent bonds. Heating can then no longer break these strong bonds, resulting in permanent (irreversible) haze.

1.2.3 Isohumulones

1.2.3.1 Formation of isohumulones

Hops serve as an essential raw material in beer brewing together with malted barley, water, and yeast. Although hops are only used in minor amounts (1-2 g.L⁻¹) with respect to malted barley (200-300 g.L⁻¹), features typical for beer are mainly determined by hop-derived compounds, thereby distinguishing beer from all other alcoholic beverages. In the brewing

process, hops are boiled for about 1.5 h with wort, a sweet tasting solution resulting from enzymic degradation of starch and proteins contained in malted barley. During boiling in the brewing kettle, humulones (**1.4**), which are present in the female hop cones (see 1.2.2.1), undergo thermal isomerization to isohumulones via an acyloin-type ring contraction.⁴⁸ Each humulone gives rise to two epimeric isohumulones, distinguished as *trans*-isohumulones (**1.1a-c**) and *cis*-isohumulones (**1.2a-c**) depending on the spatial arrangement of the tertiary alcohol function at C-4 and the prenyl side chain at C-5.⁴⁹ The denotations '*trans*' and '*cis*' indicate that these groups point to opposite faces and to the same face of the five-membered ring, respectively.

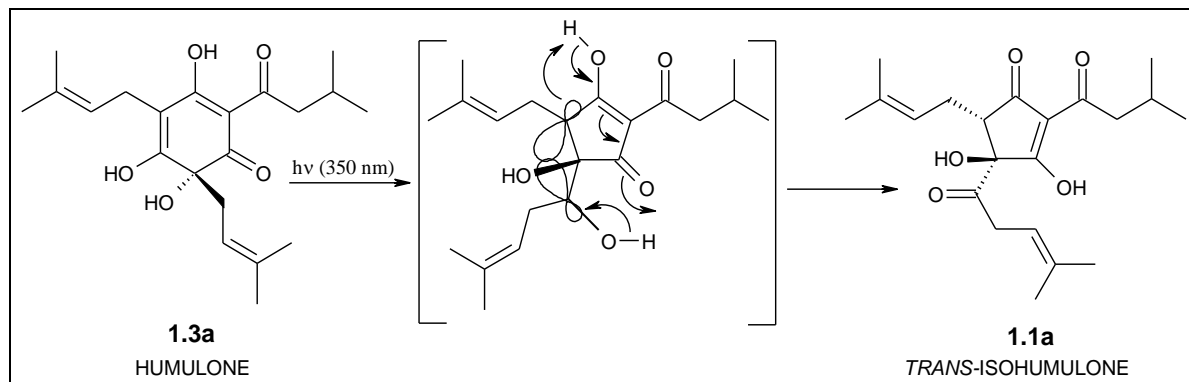


Scheme 1.9
Conversion of humulones to isohumulones.

Since there are three main humulones in hops (humulone (**1.4a**), cohumulone (**1.4b**), and adhumulone (**1.4c**)), six major isohumulones occur in beer: *trans*-isohumulone (**1.1a**) and *cis*-isohumulone (**1.2a**), *trans*-isocohumulone (**1.1b**) and *cis*-isocohumulone (**1.2b**), and *trans*-isoadhumulone (**1.1c**) and *cis*-isoadhumulone (**1.2c**). A typical ratio of *cis*-isohumulones/*trans*-isohumulones in normal brewing conditions is 68/32. *Cis*-isohumulones are thermodynamically more stable, having the two vicinal side chains in a *trans*-configuration. The utilization of humulones in brewing is rather poor, usually in the range of 25% to 35%. The main reason for this low utilization is the slightly acidic pH of the wort (5.0-5.5). Not only is the solubility of humulones very low, but also isomerization of humulones is much less efficient when compared to alkaline conditions (pH 10-11). Divalent

cations such as magnesium(II) ions also increase the isomerization rate, while the ratio of *cis*-isohumulones/*trans*-isohumulones may vary according to the experimental conditions. Thermal isomerization of the humulones can also be carried out in the solid state. The isomerization process proceeds most efficiently on heating of the solid metal salts of humulones, which are first precipitated from an aqueous alkaline solution. The best results are obtained with the calcium(II) and magnesium(II) salts of the humulones, leading to a ratio of *cis*-isohumulones/*trans*-isohumulones of ca. 85/15. Such process is used in industrial production of isomerized hop extracts.

Furthermore, it was found that humulone undergoes a regio- and selective rearrangement to *trans*-isohumulone on irradiation at 350 nm.⁵⁰ The reaction mechanism was shown to involve a (1,2)-acyl shift (oxa-di- π -methane rearrangement) from the π - π^* excited triplet state, followed by a cyclopropanol rearrangement via a SE_i mechanism with the enolic hydrogen atom acting as an internal electrophile (**Scheme 1.10**).⁵¹ The particular geometry of the bicyclic intermediate accounts for the full regio- and stereoselectivity of the photoisomerization.



Scheme 1.10

Mechanism for photoisomerization of humulone to *trans*-isohumulone.

1.2.3.2 Properties of isohumulones in beer

Isohumulones impart to a great extent the bitter taste to beer. The threshold value for bitterness in water has been estimated at ca. 6 mg.L⁻¹, which is comparable to that of quinine sulfate.⁵² The bitterness of the individual isohumulones has been shown to vary slightly with *cis*-isohumulone being rated as the most bitter constituent of a mixture of isohumulones. Typically, the concentration of isohumulones in beer varies from about 10 mg.L⁻¹ up to ca.

100 mg.L⁻¹. The perceived bitterness in beer is much less astringent than in water possibly due to the masking effect of other beer compounds including carbohydrates and proteins. Isohumulones have been implicated in both an increase in beer foam cling and lacing to the beer glass and an improvement in the overall stability of the beer head.^{53,54} According to current knowledge, isohumulones form supramolecular complexes, on the one hand with foam-active barley proteins involving both hydrogen bonding and hydrophobic interactions, and on the other hand with divalent cations (e.g. Cu(II)) via formation of stable chelates. This amphiphilic behavior creates favorable conditions for crosslinking of surface-adsorbed proteins, thus fortifying the film around foam bubbles. As a consequence, a strong foam head characterizes a well-hopped beer. Isohumulones inhibit the growth of Gram-positive bacteria and protect beer against spoilage by lactic acid bacteria (*Lactobacillus* spp. and *Pediococcus* spp.).⁵⁵ Undissociated isohumulones act as mobile carriers of ions, thereby dissipating the transmembrane pH gradient. A lower pH leads to a more efficient inhibition of microbial growth. Some strains of lactic acid bacteria were found resistant to isohumulones, but the mechanism remains elusive. On the other hand, isohumulones are quite vulnerable to light. Either direct or sensitized irradiation affords precursors for 3-methylbut-2-ene-1-thiol (MBT), the compound mainly responsible for the typical lightstruck flavor (LSF) of beer.

1.2.4 Reduced derivatives of isohumulones

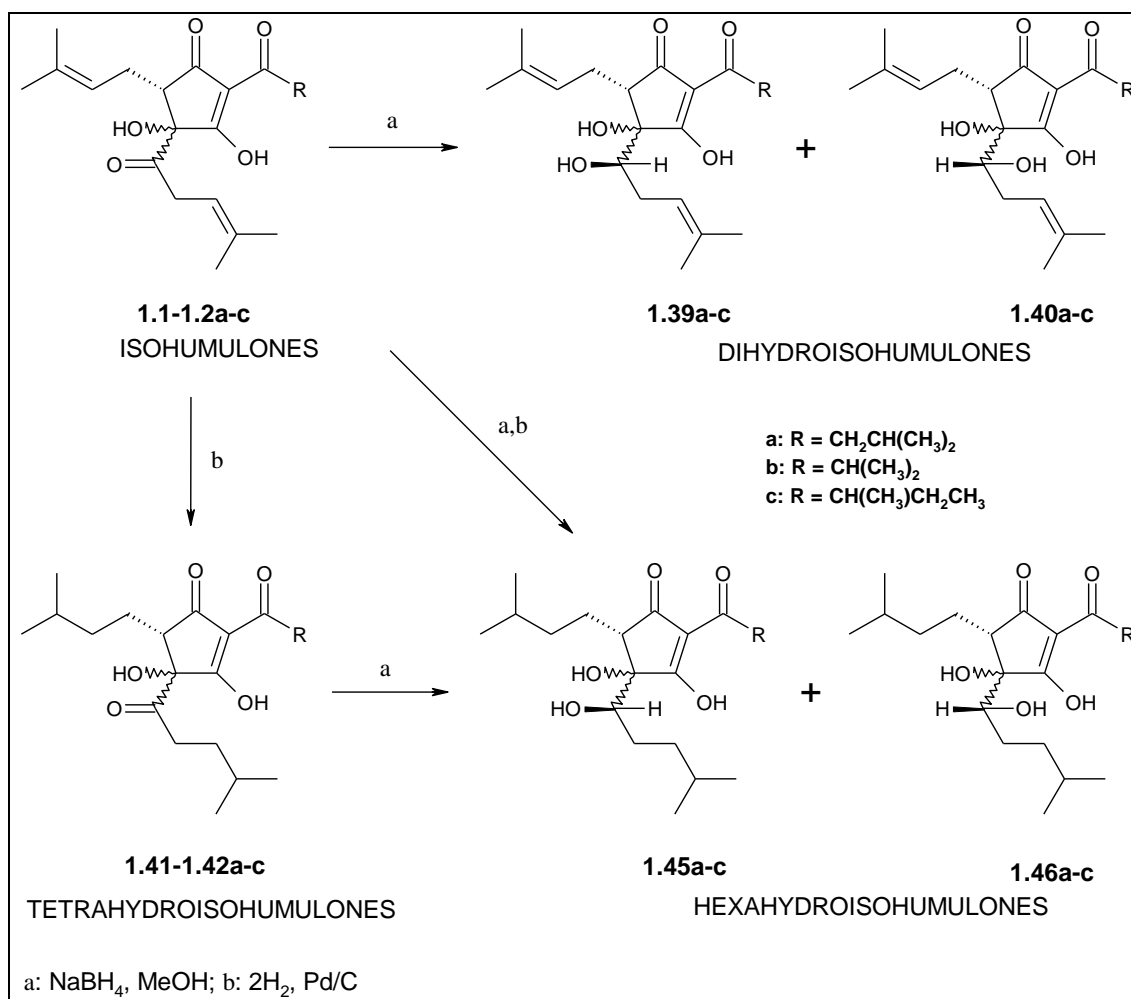
The use of whole hops in brewing has a number of disadvantages. Whole hops are very bulky, heterogeneous, prone to oxidative degradation, and the utilization of humulones is usually very poor. Solutions to these problems were provided by the development of a variety of hop products (a hop product is any form of hops in which the vegetative material cannot be recognized) including hop pellets, hop extracts, and isomerized hop products.⁵⁶ Currently, hop pellets have a market share of ca. 40% regarding the application of hops in brewing. Advantages are reflected in a much higher bulk density and an improved stability when protected from oxygen, while also an improvement in utilization of humulones is notable. Hop extracts, with a current market share of ca. 30%, are prepared by extraction of whole hops with hexane, ethanol, or carbon dioxide. Especially, liquid and supercritical carbon dioxide extracts are preferred while carbon dioxide is both environmentally benign and a natural by-product of brewing. Hop extracts are characterized by a further increase in bulk density, a significantly increased stability of hop substances, and a substantial improvement in utilization. In addition, the homogeneity results in a more consistent bitterness. To date,

isomerized hop products (or *advanced hop products*) account for ca. 10% of the use of hops and the utilization is improved by two- to threefold. The market share of isomerized hop products is expected to grow rapidly, while brewers become increasingly aware of the benefits. Furthermore, three types of reduced isomerized hop products, called *dihydroisohumulones*, *tetrahydroisohumulones*, and *hexahydroisohumulones*, are gaining widespread acceptance. The prefixes of the reduced isohumulones refer to the number of hydrogen atoms being incorporated during reduction. At first, these advanced hop products were produced in order to protect beer bottled in clear or green glass from developing a lightstruck character. But, other properties including foam enhancement and differences in relative bitterness were gradually exploited in brewing.

1.2.4.1 Dihydroisohumulones

Dihydroisohumulones (**1.39a-c**, **1.40a-c**) (also known as *rho-isohumulones*) are formed by reduction of the carbonyl group in the 4-methylpent-3-enoyl side chain using sodium borohydride (**Scheme 1.11**).^{57,58} Since formation of the secondary alcohol function is accompanied by creation of a new chiral center, two dihydroisohumulones arise from each isohumulone. Consequently, a mixture of twelve dihydroisohumulones may result from reduction of the six isohumulones. However, these mixtures are usually less complex due to particular reaction conditions leading to selectivities. For example, sodium borohydride reduction of *cis*-isohumulone produces two epimers in a ratio of 1.2/1 in isooctane/water versus 1/12 in isopropanol.⁵⁹ However, the exact composition and, also, *cis-trans* differentiation are as yet unknown.

Dihydroisohumulones are light-stable as the light-sensitive α -hydroxyketone group has been converted to a vicinal diol. Some beers produced with dihydroisohumulones may still develop a weak lightstruck character due to residual isohumulones persisting after incomplete reduction⁶⁰ or by isohumulones adsorbed to yeast if the brew is not repitched with an isohumulone-free yeast culture. The relative bitterness is only 60-80% of that of the isohumulones. Therefore, higher quantities are needed to account for a bitterness comparable to that of isohumulones. The foam-stabilizing characteristics are very similar to those of the isohumulones.

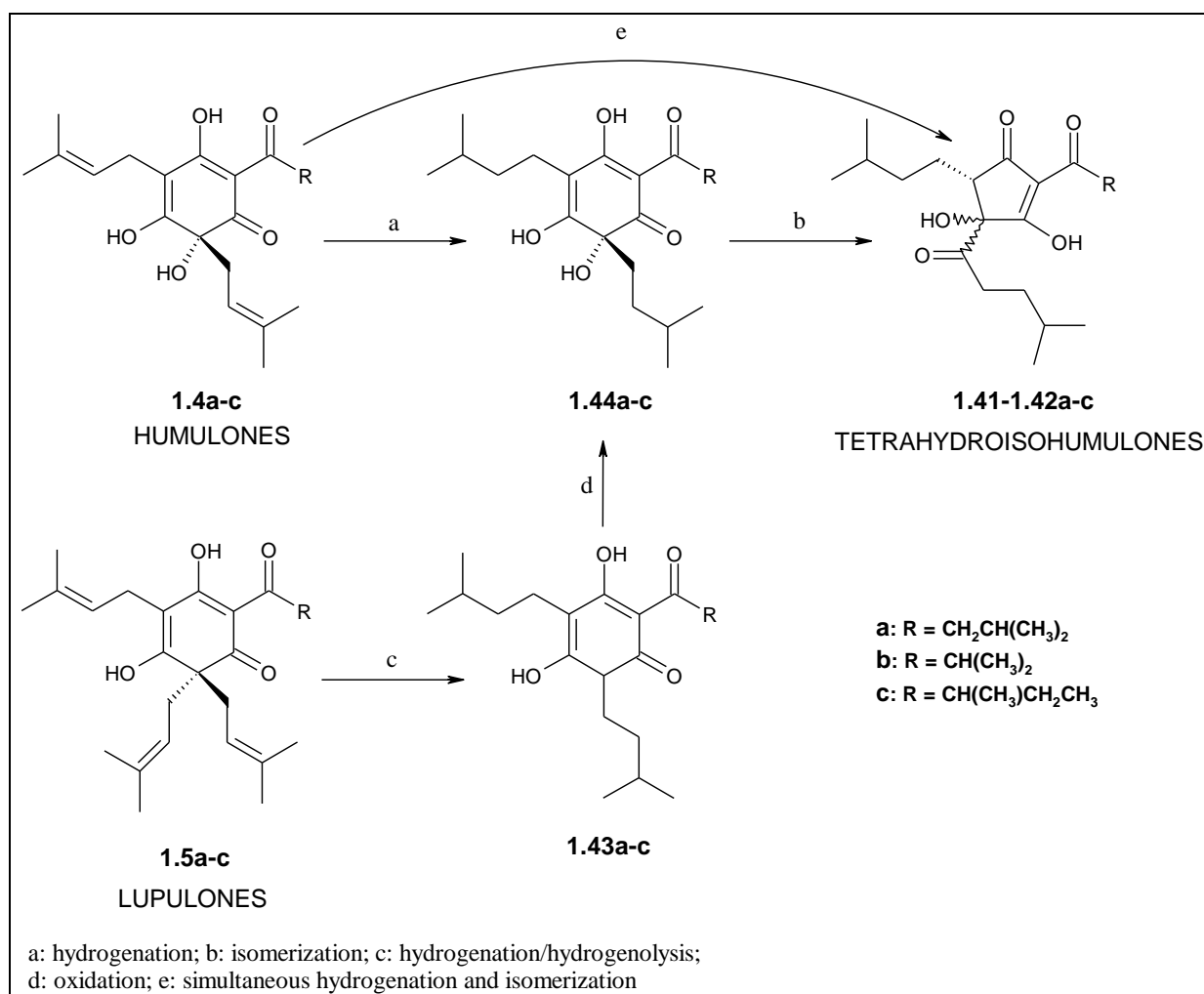
**Scheme 1.11**

Formation of reduced isohumulones.

1.2.4.2 Tetrahydroisohumulones

Three routes for the preparation of tetrahydroisohumulones (**1.41-1.42a-c**) have been reported (**Scheme 1.12**). A first procedure involves conversion of lupulones to tetrahydroisohumulones in a multistep process.^{61,62} Lupulones (**1.5a-c**) are transformed to 6-deoxytetrahydrohumulones (**1.43a-c**) via hydrogenolysis concurrent with hydrogenation. Subsequent oxidation results in formation of tetrahydrohumulones (**1.44a-c**), which are isomerized to tetrahydroisohumulones. This three-step process suffers from either inefficient oxidation or overoxidation. An alternative preparation involves isomerization of humulones (**1.4a-c**) followed by catalytic hydrogenation of the isohumulones.⁶³⁻⁶⁵ While isomerization of humulones to isohumulones is high-yielding, hydrogenation of isohumulones is not as obvious as it would appear. A third method to access tetrahydroisohumulones involves hydrogenation of humulones, followed by isomerization.⁶⁶ However, hydrogenation requires

close monitoring of the hydrogen uptake, as both overreduction and incomplete reduction may occur. On the other hand, Hay and Homiski found that humulones could be simultaneously isomerized and hydrogenated in alkaline conditions resulting in tetrahydroisohumulones in high yields (ca. 92%).⁶⁷ The reaction time was found not critical and the pH value could be easily maintained with a carbonate buffer. Consequently, this efficient one-step preparation of tetrahydroisohumulones proved suitable for large-scale production.



Scheme 1.12

Preparation of tetrahydroisohumulones from humulones and lupulones.

Saturation of the double bonds leads to enhanced hydrophobicity and diminished reactivity. Photochemical degradation may still occur, since the α -hydroxyketone group is still present, but a 3-methylbut-2-enyl radical, intervening in the formation of 3-methylbut-2-ene-1-thiol (MBT), can no longer be formed. Increased hydrophobicity as compared to

isohumulones results on the one hand in improved foam stabilizing and lacing characteristics, on the other hand in an increased relative bitterness (a factor of 1.6-1.8).⁶⁸ Advanced hop products based on tetrahydroisohumulones can be used at low levels (2-5 mg.L⁻¹) in normal beers that do not require protection against light in order to affect significantly the foam quality. While full bittering with tetrahydroisohumulones results in the appearance of an unattractive foam, a suitable combination of desired bitterness and foam stability can be found using well-balanced mixtures of dihydroisohumulones and tetrahydroisohumulones.⁶⁹

1.2.4.3 Hexahydroisohumulones

Hexahydroisohumulones (**1.45a-c**, **1.46a-c**) are accessible by a combination of processes used in the preparation of dihydroisohumulones and tetrahydroisohumulones (**Scheme 1.11**). Simultaneous or successive reduction of the carbonyl group in the side chain at C-4 and catalytic hydrogenation of the double bonds in the prenyl side chains of the isohumulones afford hexahydroisohumulones. The mixture of hexahydroisohumulones may consist of twelve isomers and homologs, but real mixtures are usually less complex due to selectivities exerted during the reactions. Consequently, the exact composition and, also, *cis-trans* differentiation are as yet unknown.

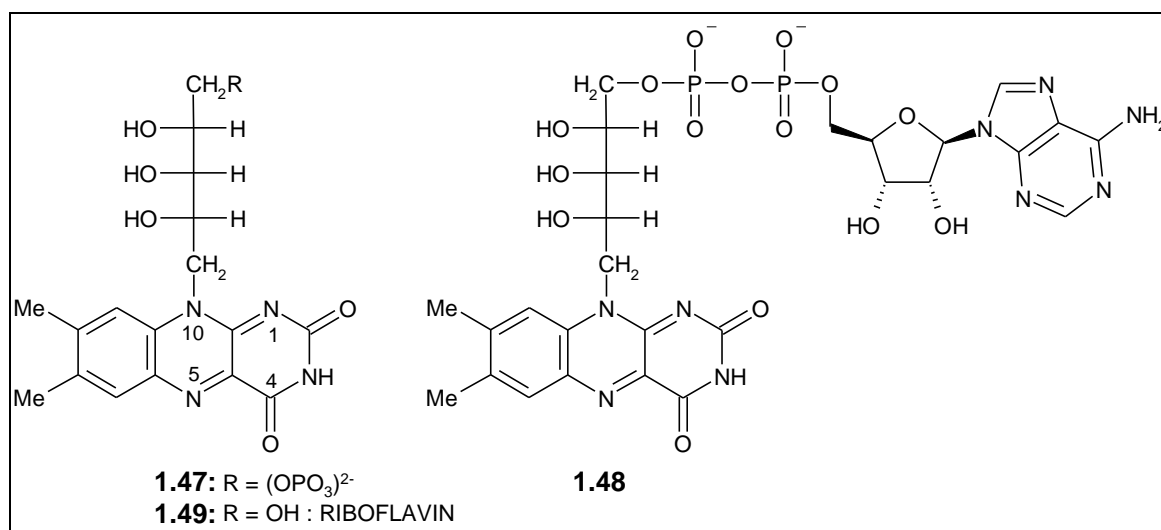
Hexahydroisohumulones are light-stable and they combine the features associated to dihydroisohumulones and tetrahydroisohumulones. The relative bitterness is weakly increased with respect to isohumulones, while foam-enhancing characteristics are even further improved relative to those of tetrahydroisohumulones. Low solubility and high cost hamper applications in the brewing practice.

1.3 ROLE OF FLAVINS

1.3.1 Introduction

Flavins are based on a nitrogen-containing heterocyclic system, called 7,8-dimethylisoalloxazine or 7,8-dimethylbenzo[g]pteridine-2,4-(3*H*,10*H*)-dione. The most common natural flavins are flavin mononucleotide (FMN) (**1.47**), flavin adenine dinucleotide (FAD) (**1.48**), and riboflavin (RF, vitamin B₂) (**1.49**) (**Scheme 1.13**). The denotation ‘flavin’

refers to the yellow color of the conjugated ring system. Flavins are reputed for their chemical and biological versatility.⁷⁰



Scheme 1.13
 Structures of the most important flavins.

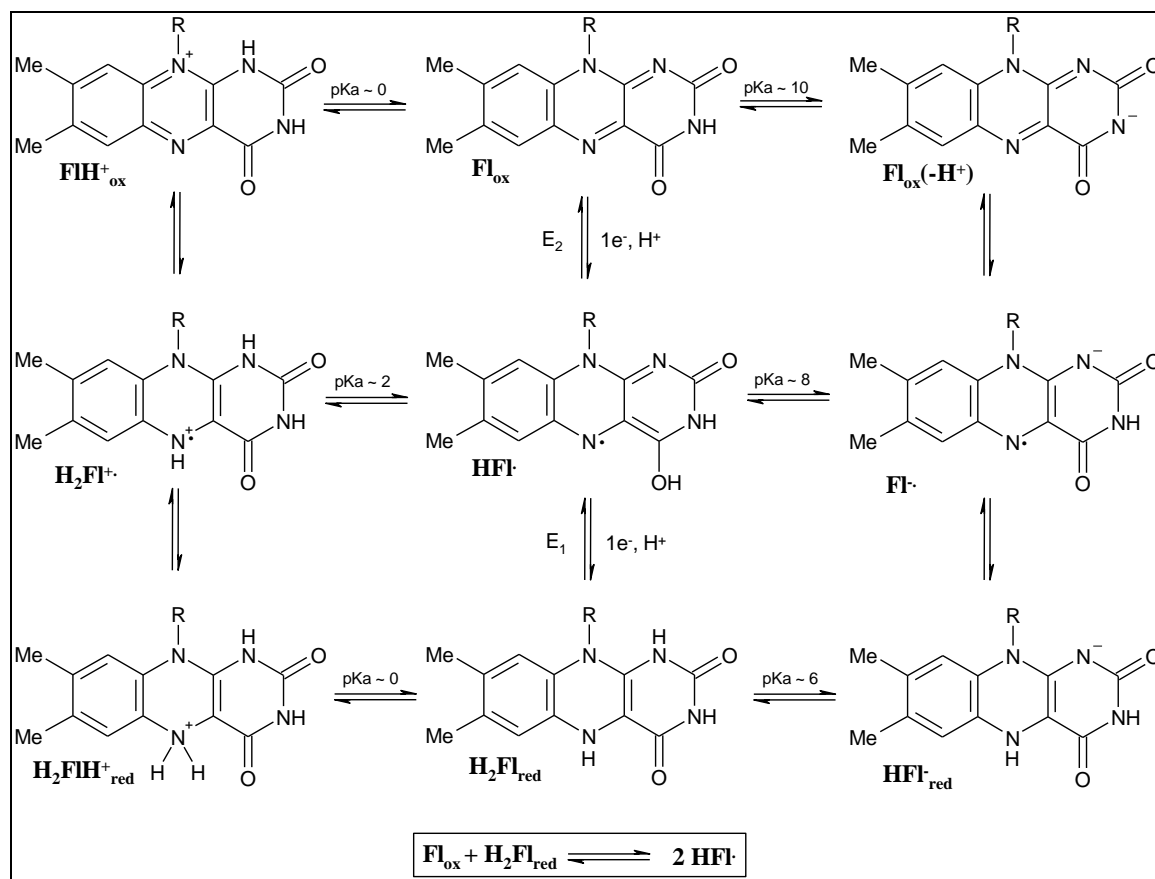
1.3.2 Biological role of flavins

Flavins, while being capable to induce one- and two-electron transfer processes, were found to play a pivotal role in coupling the two-electron oxidation of most organic substrates to the one-electron transfers in the respiratory chain.⁷¹ In addition, they can function as electrophiles and nucleophiles.⁷² Flavins are thought to contribute to oxidative stress through their ability to produce superoxide radicals from oxygen,⁷³⁻⁷⁵ but, at the same time, flavins are frequently involved in reduction of hydroperoxides, derived from oxygen-derived radical reactions. Furthermore, flavoproteins are important in soil detoxification via hydroxylation of various aromatic compounds.⁷⁶ Flavins are also involved in light-production by bioluminescent bacteria⁷⁷ and they are intimately connected to light-initiated reactions such as plant phototropism⁷⁸ and nucleic acid repair processes.⁷⁹⁻⁸¹ Recent reports also link flavins to programmed cell death.⁸²⁻⁸³

1.3.3 Chemical properties of flavins

The ability to participate in one-electron transfer reactions implies the existence of semiquinone-type oxidation states. In free solution, i.e. when not enzyme-bound, a mixture of oxidized flavins (**Fl_{ox}**) and reduced flavins (**Fl_{red}**) very rapidly leads to an equilibrium,

containing also flavin radicals (**HFI[•]**) (**Scheme 1.14**). In free flavins, the equilibrium is shifted to the left, and, at pH 7, only 5% of the radical form is present in an equimolar mixture of oxidized and reduced flavins.



Scheme 1.14
Redox and acid-base equilibria of flavins.

In addition, redox forms of 7,8-dimethylisoalloxazines occur as protonated species.⁸⁴ The redox potentials of free FMN at pH 7.0 and 20°C were found to be -0.207 V (vs NHE) for an overall two-electron reduction (E_m or midpoint redox potential), -0.313 V (vs NHE) for reduction of oxidized flavin to the semiquinone (E_2), and -0.101 V for reduction of the semiquinone to the hydroquinone (E_1).⁸⁵ Redox potentials measured for FAD and riboflavin at pH 7.0 were found similar to those of FMN.

Physicochemical properties of a flavin moiety in flavoproteins including pKa values and midpoint redox potentials depend strongly on the host protein. For example, the free semiquinone of FMN can exist in a neutral or an anionic form with a pKa value of ca. 8.5. On binding to flavodoxins, the FMN semiquinone is thermodynamically stabilized and the pKa

value is shifted by ca. four units to a higher value with accompanying large changes in the redox potentials of one-electron reductions.⁸⁶ Furthermore, significant spectral differences exist between the various flavin oxidation states. Therefore, it is feasible to monitor the events occurring in enzyme catalysis using the flavin itself as a reporter (e.g. glucose oxidase) (Figure 1.6).⁸⁷

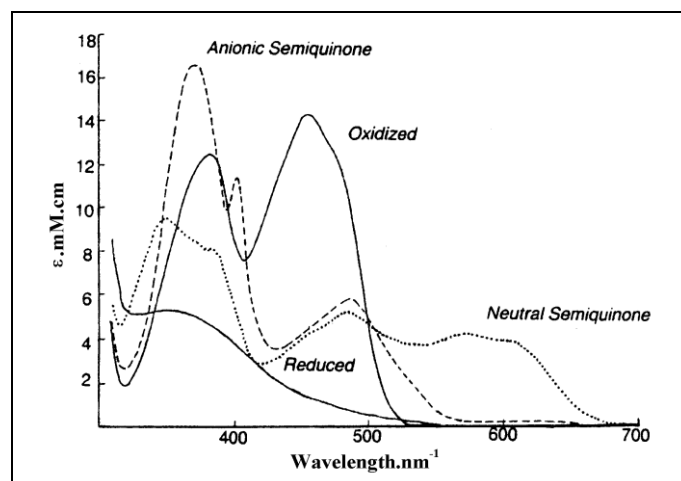


Figure 1.6
UV/Vis spectra of various flavin oxidation states.

1.3.4 Photophysical and photochemical properties of flavins

The absorption spectrum of a flavin such as RF in aqueous solution consists of four structureless peaks centered at 446 nm, 375 nm, 265 nm, and 220 nm, respectively. All four absorption maxima possess high molar extinction coefficients ($> 10^4 \text{ M}^{-1} \text{ cm}^{-1}$) indicative of $\pi \rightarrow \pi^*$ transitions. Generally, $n \rightarrow \pi^*$ transitions are of low probability due to symmetry and/or overlap restrictions, however, they are potentially intense in flavins due to the lack of molecular symmetry. Still, the balance of evidence indicates that any $n \rightarrow \pi^*$ transition is masked by the much more intense $\pi \rightarrow \pi^*$ transitions.⁸⁸

Flavins exhibit a bright yellow fluorescence ($\lambda_{\text{max}} = 520 \text{ nm}$) in aqueous solution. An essentially identical quantum yield of fluorescence (Φ_f) of 0.26 has been reported for RF and FMN in dilute aqueous solution at pH 7.⁸⁹ The same values for Φ_f are obtained irrespective of the excitation wavelength (260-500 nm).⁹⁰ The wavelength independence of Φ_f indicates that excitation to higher excited states ($S_{n>1}$) is followed by extremely rapid internal conversion to the first excited singlet state (S_1) without loss of energy due to emission from the upper

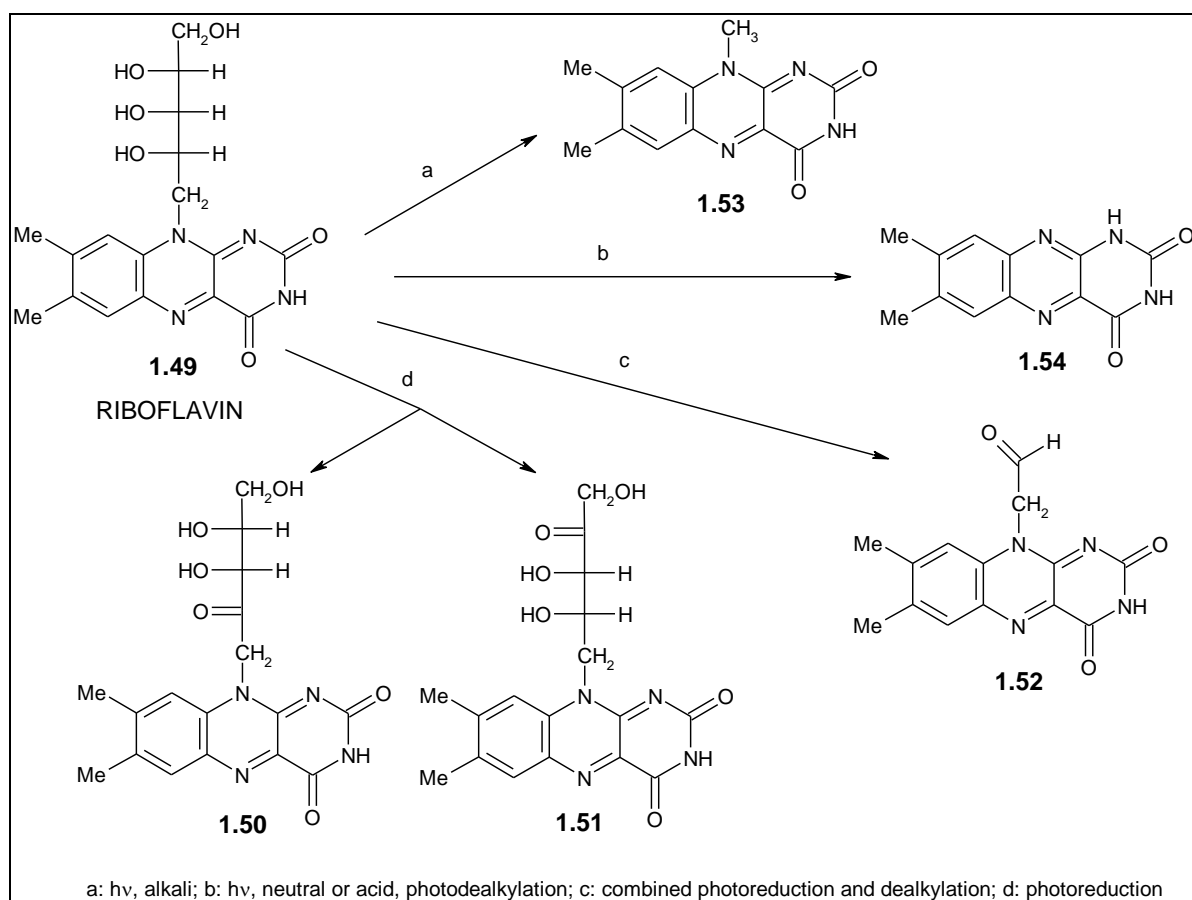
excited states or from intersystem crossing from higher excited singlet states to excited triplet states ($T_{n \geq 1}$). Furthermore, Φ_f was found to depend strongly on the solvent polarity with $\Phi_f = 0.26$ in aqueous solution, but rising to 0.47 in acetonitrile.⁹¹ Direct measurements of the radiative lifetime of the lowest excited singlet state using time-resolved techniques yield values of ca. 5 ns for RF, and ca. 2 ns for FMN and FAD.⁹²

Flavins exhibit an orange-red ($\lambda_{\max} = 610$ nm) phosphorescence at low temperatures (< 150 K).⁹³ The quantum yield of phosphorescence (Φ_p) is generally quite low (e.g. $\Phi_p = 0.0012$ in ethanol at 77 K).⁹⁴ This contrasts with photolysis data, which show that intersystem crossing is very efficient ($\Phi_{ISC} = 0.7$), at least at room temperature and in fluid solution.⁹⁵ The lifetime of phosphorescence at 77 K is in the range of 0.1 to 0.2 s, the exact value depending on the nature of the flavin and on the solvent matrix.⁹⁴ In fluid solution at room temperature, the lifetimes of triplet-excited flavins are much shorter (10-100 μ s).⁹⁶ Using both low-power Xe-flash and high-power laser photolysis, Yoshimura and Ohno showed that the excited triplet state of lumiflavin was quenched by both the excited triplet state and by radicals derived thereof.⁹⁷

In view of the conflicting high efficiency of intersystem crossing and low quantum yield for phosphorescence, it would appear that the lowest excited triplet state decays predominantly by non-radiative internal conversion. However, a large variety of photochemical reactions may occur from the excited triplet state, while, also, the excited singlet state is involved in a more restricted number of photochemical reactions. Flavin photochemistry can be conveniently divided into three pathways: photoreduction, photodealkylation, and photo-addition. It should be realized, that, depending on the structure of the flavin and the reaction conditions, some or all of the various photoreactions may occur simultaneously.⁸⁴

Both intermolecular and intramolecular photoreductions are known. The intermolecular reaction occurs in the presence of a wide range of substrates including amino acids, α -hydroxycarboxylic acids, thiols, aldehydes, and unsaturated hydrocarbons.⁹⁸⁻¹⁰⁰ Intermolecular photoreductions yield either free 1,5-dihydroflavins or alkylated derivatives. The intramolecular photoreduction involves dehydrogenation of the hydroxyalkyl side chain at the *N*-10 position to yield a variety of keto or aldehyde functions in the side chain with or

without partial loss of some carbon atoms.¹⁰¹ Riboflavin (RF) (**1.49**) has been extensively studied and presents a complex product distribution including 2'- and 4'-keto derivatives (**1.50** and **1.51**, respectively), together with formylmethyl flavin (**1.52**), which is formed via combined intramolecular photoreduction and dealkylation (**Scheme 1.15**). On irradiation in alkali formation of lumiflavin (**1.53**) was observed. Furthermore, quenching studies revealed that intramolecular photoreduction may proceed from both the excited singlet and triplet states.



Scheme 1.15
Photoreactions of riboflavin.

Photodealkylation is strictly an intramolecular reaction yielding an alloxazine and an alkene. Little is known about the mechanism, but two alternative processes have been proposed. The first mechanism involves homolytic fission of the *N*-10/*C*-1' bond in the biradical derived from hydrogen abstraction of the α -CH bond in the side chain by *N*-5.¹⁰² The alternative mechanism proceeds by a synchronous process, which does not involve radical intermediates. The excited singlet state is thought to be the major intermediate

involved in photodealkylation of riboflavin, since triplet-state quenchers do not inhibit formation of lumichrome (**1.54**) (Scheme 1.15).¹⁰¹

Intermolecular photoaddition has been reported where ROH is the solvent (R = H or alkyl) and the solvent residue 'RO' adds to the C-6 or C-9 positions of the benzenoid subnucleus.¹⁰³ Intramolecular photoaddition to the C-9 position occurs if a free hydroxy group is present at the C-2' position.¹⁰⁴ Furthermore, a number of other photophysical phenomena and photochemical reactions have been reported including photoelectron ejection,¹⁰⁵ energy transfer,¹⁰⁶ cyclization of flavins possessing a phenyl substituent at *N*-10,¹⁰⁷ photohydrolysis and ring cleavage reactions,¹⁰⁸ and photodehalogenation.¹⁰⁹

In the presence of oxygen, flavins sensitize or catalyze the oxidation of various substrates including amino acids,¹¹⁰ proteins,¹¹¹ DNA and nucleotides,¹¹² and lipids.¹¹³ Oxidation of substrates may proceed via a radical (or Type I) mechanism involving electron abstraction from the substrate, or via a non-radical (or Type II) mechanism involving energy transfer from triplet-excited flavin to oxygen in the ground state thereby converting triplet oxygen to the highly reactive singlet oxygen.¹¹⁴ Furthermore, photooxidations may also proceed by direct triplet-triplet exergonic energy transfer from triplet-excited flavin to a substrate. Such mechanism may operate, for example, in the flavin-sensitized photooxidation of retinal,¹¹⁵ bilirubin,¹¹⁶ and stilbenes.¹¹⁷

1.3.5 Flavins in beer

Quantitative analyses using HPLC with fluorescence detection have shown that riboflavin (RF) is by far the most important flavin in beer with concentrations ranging from 100 to 600 $\mu\text{g.L}^{-1}$.¹¹⁸ Flavin adenine dinucleotide (FAD) was detected in all beers at concentrations of 10-70 $\mu\text{g.L}^{-1}$, while flavin mononucleotide (FMN) was found in trace amounts in few beers.

Barley contains RF (1.2-1.5 mg.kg^{-1}) and the concentration increases during malting, especially at higher germination temperatures.¹¹⁹ RF is extracted quantitatively into wort and survives wort boiling. Furthermore, RF is also produced by yeast with the production rate being proportional to yeast growth. On the other hand, part of RF is destroyed during the stationary phase of the yeast as a result of enzymic breakdown.

1.4 ROLE OF THE SULFUR SOURCE

The source of the sulfur atom in 3-methylbut-2-ene-1-thiol (MBT), has not yet been identified. Experiments, in which beer was dialyzed against water in cellophane membranes, indicated that the donor molecules are of both high and low molecular weight. Thus, proteins, polypeptides, as well as free amino acids may take part in the lightstruck reaction. Cysteine and cystine have been implicated by sensory analysis in the formation of lightstruck flavor (LSF) in model systems.²¹ In contrast, Blockmans *et al.* detected no MBT after irradiation of a solution containing cysteine, isohumulones and riboflavin.¹²⁰ It is conceivable that differences in the redox potentials of the model systems contributed to the discrepancy of the results.

Previous work implicated dihydrogen sulfide as a possible sulfur source, but MBT is only formed from dihydrogen sulfide in a highly reducing medium. Sakuma *et al.* showed that dihydrogen sulfide could contribute to the formation of MBT, when high concentrations ($> 50 \text{ mg.L}^{-1}$) of ascorbic acid were present in addition to isohumulones and RF.¹⁹ In commercial beers, cleavage of dihydrogen sulfide to a thiol radical may be less significant than decomposition of sulfur-containing compounds. It has been suggested that sulfur-containing amino acids and proteins decompose to a thiol radical in RF-photosensitized reactions.¹⁹ On the other hand, reactive oxygen species such as hydroxyl and hydroxyethyl radicals have been shown to generate thiyl radicals from thiol-containing compounds including cysteine.²¹

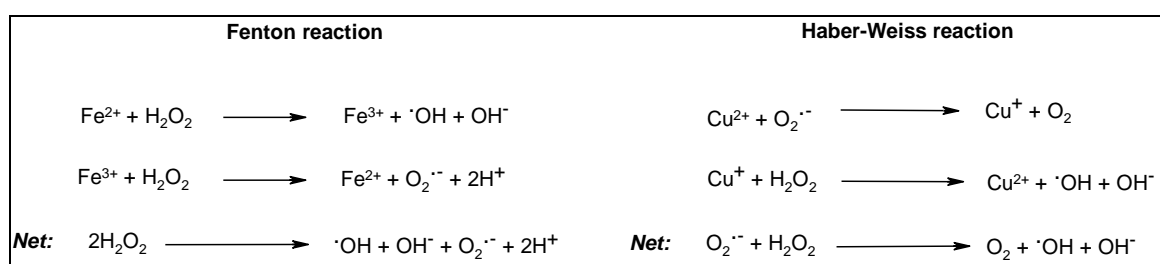
1.5 FLAVOR STABILITY OF BEER

Beer flavor is not only subject to deterioration by the influence of light. Next to light-induced formation of MBT as a 'skunky' thiol, significant flavor changes can be observed during ageing of beer. The quality of bitterness decreases¹²² and chemical reactions of compounds in beer produce an oxidized flavor.¹²³ A variety of radical reactions may lead to formation of unsaturated aliphatic aldehydes containing six to ten carbon atoms such as *trans*-2-nonenal, which is largely responsible for the cardboard flavor of aged beers,¹²⁴ whereas formation of vicinal diketones and furan derivatives results in 'buttery' off-flavors.

Formation of aldehydes in beer is complex.¹²⁵ During malting and mashing, aldehydes can be formed via oxidation of fatty acids, either through combined actions of lipoygenase and

lipase or via auto-oxidation to form hydroperoxides, which can be converted to staling aldehydes. Aldehydes could also be formed via Strecker degradation of amino acids and these could combine to form long-chain aldehydes via aldol condensations. During fermentation, the reducing power of yeast converts most aldehydes remaining in the wort post-boiling to their corresponding alcohols.¹²⁶ During storage, aldehydes may be formed via melanoidin-mediated oxidation of higher alcohols, oxidation of isohumulones, Strecker degradation of amino acids, auto-oxidation of fatty acids or aldol condensations of short-chain aldehydes.

It is clear, that maintaining a low temperature during storage of beer and minimization of oxygen during brewing result in improvements in flavor stability. These facts form the basis of flavor stability tests using aerobic forced ageing at elevated temperatures. The increase in the concentration of carbonyls during beer ageing has been linked to the presence of oxygen and metal ions.¹²⁷ Free radicals have been identified by electron spin resonance spectroscopy as reaction intermediates in oxidation processes occurring in beer during aerobic forced ageing. It was demonstrated that addition of iron salts and hydrogen peroxide accelerated formation of radicals in beer. Moreover, addition of catalase, which destroys hydrogen peroxide, or addition of chelating agents that bind metal ions proved to slow down production of radicals.¹²⁷ These results provide evidence that Fenton-type oxidations in beer can take place in the presence of oxygen serving as a precursor of hydrogen peroxide, which subsequently reacts with iron (Fenton reaction) or copper (Haber-Weiss reaction) ions to yield hydroxyl radicals (**Scheme 1.16**).



Scheme 1.16
Fenton and Haber-Weiss reactions leading to hydroxyl radicals.

Uchida and Ohno used spin traps to detect radicals in beer as spin adducts and they assigned the trapped radicals as hydroxyl radicals.¹²⁸ On the other hand, Andersen and Skibsted found the 1-hydroxyethyl radical to be the quantitatively most important radical.¹²⁹ This can be readily understood, while an hydroxyl radical reacts at essentially diffusion-

controlled reaction rates with most organic compounds and while ethanol is the beer component found in the highest concentration besides water. Furthermore, by monitoring the concentration of the spin adducts in function of time during aerobic forced ageing, it was observed that the spin adducts were not immediately formed on starting the forcing test. Initially, only a negligible amount of spin adducts was detected, while, after a certain period of time, called the lag phase, the amount of spin adducts began to increase linearly with time. The length of the lag phase of fresh beer has been shown to correlate with the flavor stability of beer and the ESR technique is quite appropriate to predict the stability of beer.¹³⁰ The length of the lag phase has also been referred to as the endogenous antioxidant activity of beer, while it is the result of competition between the actions of prooxidative and antioxidative components of beer. The method, therefore, provides an excellent way to examine potential antioxidants in beer.¹³¹ Furthermore, this assay observes both the time it takes before the natural antioxidants are exhausted (the lag phase) and the rate at which radicals are formed in the absence of antioxidants. Until now, only sulfite was able to delay formation of radicals, whereas phenolic compounds such as phenolic acids, catechin, epicatechin, and proanthocyanidin dimers had no effect on the formation of radicals. Ascorbate, cysteine, and cysteamine, on the other hand, were found to be prooxidants. In order to be effective under aerobic forced ageing tests, antioxidants must be able to either scavenge peroxides or trap metal ions. The effectiveness of sulfite is probably a consequence of its two-electron non-radical producing reaction with peroxides.¹³²

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OBJECTIVES

Our current understanding of formation of the lightstruck flavor in beer dates from the original reports by Kuroiwa et al. in the early sixties. A formal mechanism involves photosensitized degradation of isohumulones by riboflavin and subsequent trapping of a 3-methylbut-2-enyl radical by a thiol radical derived from suitable sulfur source (e.g. cysteine) to give 3-methylbut-2-ene-1-thiol. All relevant data collected to date do not contradict the Kuroiwa premise. Although the key components are known for more than three decades, very few investigations have reported on the mechanisms of interaction between these components.

This Ph.D. project aimed at obtaining a comprehensive understanding of the mechanism of formation of the lightstruck flavor in beer. The issue was approached by an in-depth investigation of the interacting components on a molecular level using simple model systems, consisting of isohumulones, dihydroisohumulones or tetrahydroisohumulones in the presence or absence of riboflavin or flavin mononucleotide as a sensitizer.

Unraveling a photochemical mechanism implies studies in various time domains. Thus, model systems must be subjected to both photostationary irradiation and photodynamic flash-initiated experiments. Information on the nature of intervening photo-excited states and intermediates, along with mechanistic features, can be retrieved from time-resolved electron paramagnetic resonance and laser-flash photolysis transient absorption spectroscopy. Characterization and identification of prevalent photoreaction products by spectroscopic techniques should allow to elaborate relevant reaction schemes to account for formation of key compounds.

The objective was, in a first phase, to undertake a detailed study of the photolability of isohumulones, dihydroisohumulones, and tetrahydroisohumulones. In a second phase, the purpose was to investigate the interaction between isohumulones, dihydroisohumulones or tetrahydroisohumulones with riboflavin or flavin mononucleotide on sensitized irradiation.

It was advanced that interpretation of photostationary and photodynamic experiments would prove that the events leading to formation of the lightstruck flavor differ according to the irradiation conditions. Thus, the principal target was to elucidate the (photo)reaction mechanisms operating either under direct UV irradiation or under visible light irradiation in the presence of a sensitizer. Knowledge of intricate mechanistic details should then allow to find innovative and proprietary ways to prevent formation of the lightstruck flavor in beer.

**DIRECT IRRADIATION
WITH UV LIGHT**

CHAPTER 2

TIME-RESOLVED ELECTRON PARAMAGNETIC RESONANCE OF ISOHUMULONES AND REDUCED DERIVATIVES UNDER DIRECT IRRADIATION WITH UV LIGHT

2.1 ELECTRON PARAMAGNETIC RESONANCE

2.1.1 General introduction

The technique of electron paramagnetic resonance (EPR) spectroscopy is very similar to the more familiar nuclear magnetic resonance (NMR) technique. While NMR involves the interaction of electromagnetic radiation in the radiofrequency range with the magnetic moments of nuclei, EPR is based on the interaction of microwave radiation with the magnetic moments of electrons.^b Each electron possesses an intrinsic magnetic dipole moment that arises from its spin. In most systems, electrons occur in pairs such that the net moment is zero. Hence, only species that contain one or more unpaired electrons possess the net spin moment necessary for suitable interaction with an electromagnetic field. Typical systems that are studied by EPR include free radicals (containing one unpaired electron), triplet-state systems and biradicals (containing two unpaired electrons), transition metal ions (including actinide ions), and point defects in solids.¹

In order to characterize free radicals in liquid solution, two magnetic parameters are normally measured. These are the g-factor (chemical shift) and the electron-nuclear hyperfine interaction, which for protons is written as a_H . The g-factor for a free electron (g_e), determined to be 2.002319304386, is one of the most accurately known physical constants. Most organic radicals centered on C, N, O, S or P atoms have a g-factor, which varies only some ± 0.003 units from the value for a free electron. This follows from the fact that an electron in free radicals can move more or less freely over an orbital encompassing the whole molecule and the electron is not confined to a localized orbital between two atoms in a molecule. Therefore, its behavior is very much the same as an electron in free space. For the abovementioned

^b EPR is also frequently referred to as electron spin resonance (ESR), while, in most cases, the absorption is linked primarily to the electron-spin angular momentum. The denotation 'EPR' takes into account contributions from an electron orbital as well as from a spin angular momentum.

radicals, the g -factor on itself is not very informative and further information can be obtained from the electron-nuclear hyperfine interaction, which arises through coupling of the unpaired electron with neighbouring nuclear spins in much the same way as the coupling between nuclear spins in NMR. In general, a nucleus with spin I will split the resonance line of an electron into a multiplet with $2I + 1$ lines of equal intensity. Hyperfine interactions between carbon-centered radicals and protons are on the order of 1-40 Gauss, giving spectral widths (in frequency units) that range from 10 MHz to 300 MHz for typical organic radicals. These couplings are larger by a factor of ca. 10^6 with respect to nucleus-nucleus coupling, which can be accounted for by the fact that an electron can approach a nucleus more closely than another nucleus and will, therefore, interact more strongly with the nucleus. The most important factor influencing the magnitude of electron-nucleus coupling is the length of time, which the electron spends in the vicinity of the coupled nucleus or, in other words, the electron density at the nucleus (more correctly, the spin density rather than the physical density). Therefore, EPR can be used to build up a qualitative picture of electron distribution within a radical.

The resonant frequency of most organic radicals in the microwave region of the electromagnetic spectrum is equal to ca. 9.5 GHz with an applied external field of 3.4 kGauss. The 9.5 GHz, called the X-band, is the standard frequency for most commercial EPR spectrometers due to the availability of inexpensive X-band microwave components used in radar. The EPR signals (either absorption or emission) are detected using a microwave bridge circuit coupled to a resonant cavity with the sample in the center, positioned such that the microwave magnetic field is at a maximum for EPR sensitivity and, concurrently, that the microwave electric field is at a minimum. In a conventional EPR experiment, which is referred to as steady-state EPR (SSEPR), the resonant cavity is constantly irradiated (continuous wave or CW) during the entire data collection process and a stationary concentration of radicals of 10^{-8} M is readily detectable. In SSEPR, the transitions are affected by sweeping an external magnetic field through each resonance at constant frequency. Resonance occurs when the energy difference for allowed EPR transitions (between energy levels with a corresponding inverted electron spin, but not an inverted nuclear spin) matches the frequency. Furthermore, the combination of a modulated external field (usually at a frequency of 100 kHz) and phase-sensitive detection are used to increase the signal-to-noise (S/N) ratio and the spectral resolution. The resulting spectra, therefore, have typical first-derivative line shapes.

As a consequence of the field modulation, the time response of the EPR spectrometer becomes limited and, therefore, species with chemical lifetimes less than 40 μs are difficult to detect. Organic free radicals are, with few exceptions, short-lived species at room temperature with chemical lifetimes ranging from ns to ms. Thus, many paramagnetic organic compounds cannot be detected by SSEPR. Possibilities to overcome this problem include detection at lower temperatures (below -50°C) with inherent longer lifetimes or by continuous, intense irradiation in order to generate a detectable steady-state concentration of radicals.

2.1.2 Time-Resolved Electron Paramagnetic Resonance (TREPR)

A more elegant method to achieve the time resolution needed for detection of radicals with short lifetimes is based on pulsed production of radicals and synchronized gated detection. While radicals are often produced using light, pulsed production of radicals can readily be accomplished with a pulsed laser source. Connecting the EPR signal from the microwave bridge to a boxcar-gated integrator allows the signal to be trapped and stored on the sub- μs time scale. This method is referred to as *time-resolved electron paramagnetic resonance* (TREPR), but it is not a pulsed technique in the same sense as modern NMR spectroscopy. While the microwave excitation of the spin levels is continuous during the TREPR experiment, it is more appropriate to refer to this technique as time-resolved continuous-wave electron paramagnetic resonance (TREPR (CW)).² A schematic overview of the apparatus is presented in **Figure 2.1**. It should be noted that pulsed EPR can be used also (e.g. Fourier-transform EPR), but such technique is considerably more expensive and complex, and suffers from limitations imposed by the pulsing technology.^{3,4}

The timing sequence for a TREPR (CW) experiment is shown in **Figure 2.2**. After the trigger, the first gate or boxcar samples the dark EPR signal directly from the output of the microwave bridge to provide a baseline. The second gate or boxcar opens at a set delay time τ after the flash and samples the altered EPR signal. The light-induced EPR signal is then given as the difference between the two gates. The time resolution of the experiment is determined by the width of the gates (typically 100 ns) and common delay times (τ) are 0.1-10 μs .

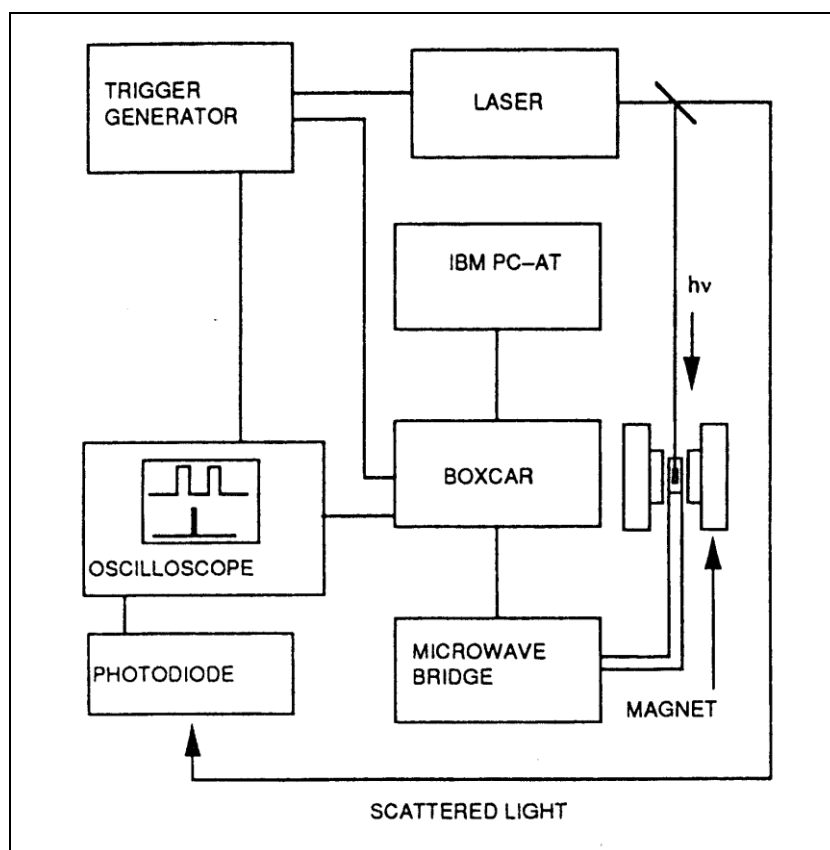


Figure 2.1
Experimental set-up for the TREPR (CW) technique.

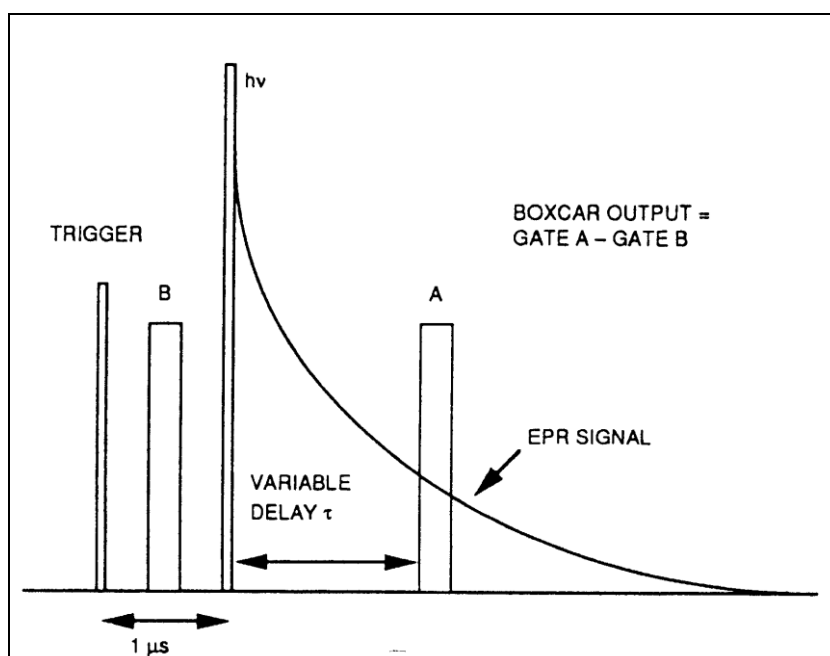


Figure 2.2
Timing sequence for a TREPR (CW) experiment.

Because the aim is to detect radicals with short lifetimes, the field modulation, limiting the time response, must be bypassed. As a consequence, TREPR suffers from a drastic reduction in sensitivity versus SSEPR, but this is largely compensated for by the use of the boxcar as a signal averager and, more importantly, by the phenomenon of *chemically induced dynamic electron polarization* (CIDEP).⁵ Chemically induced spin polarization processes, which are common in photochemical reactions, decay with T_1 values on the order of 1-10 μ s, and are, thus, readily observable in TREPR, if the radicals do not experience unusually fast spin relaxation. The presence of CIDEP results in a non-Boltzmann population of the spin states, provides an amplification of the signal in most radical reactions, and leads to unusual intensity patterns in the ensuing EPR spectra with some transitions in emission instead of absorption. There are four distinct mechanisms known to produce such polarization, and a significant amount of kinetic and magnetic information can be obtained by studying these phenomena.

If nuclear spin-dependent electron wave function evolution in a geminate radical pair occurs on the same time scale as diffusive encounters between the radicals, multiplet polarization of the spins ensues. This is referred to as the *radical pair mechanism* (RPM) of CIDEP⁶ and, for triplet precursors, it gives an emissive/absorptive (E/A) pattern about the center of the spectrum (A/E for singlet precursors). A second mechanism, called the *radical-triplet pair mechanism* (RTPM),⁷ is closely related to RPM, but it involves interaction of a photoexcited triplet state with a doublet state radical. Another CIDEP mechanism that is only observable with triplet precursors is the *triplet mechanism* (TM).⁸ It arises from selective population of the triplet sublevels of the precursor during intersystem crossing. TM is always observed as a net effect, either absorptive (A) or emissive (E) depending on the sign of the zero-field splitting in the precursor triplet state. The last mechanism of CIDEP is called the *spin-correlated radical pair mechanism* (SCRPM),⁹ as it arises when a non-zero spin-spin interaction is operating such as exchange or dipolar coupling between two unpaired electrons at the time of the measurement. Examples of systems where SCRPM spectra are observed include radical pairs confined to micelles, covalently linked donor-acceptor complexes, the photosynthetic reaction center, and flexible alkane chain biradicals.

2.2 TREPR OF ISOHUMULONES AND REDUCED DERIVATIVES

2.2.1 Observation of TREPR signals

Figure 2.3A shows the X-band (9.5 GHz) TREPR spectrum obtained at a delay time of 0.3 μ s after laser photolysis at 308 nm of a toluene/methylcyclohexane (1/1, v/v) solution of *trans*-isohumulones (**2.1a-c**; **2.1a**: 54%, **2.1b**: 32%, **2.1c**: 14%) (ca. 10 mM). The fact that a strong emissive TREPR signal is detected indicates that free radicals are, indeed, produced when UV light strikes isohumulones. The net emission is generated via the triplet mechanism of CIDEP. The strong intensity in the center of the spectrum and the weak signals on the edges indicate the presence of at least two different radical species with significant electron-nuclear hyperfine interactions.

Laser photolysis at 308 nm of a solution of *trans*-tetrahydroisohumulones (**2.2a-c**; **2.2a**: 46%, **2.2b**: 42%, **2.2c**: 12%) in methylcyclohexane (ca. 10 mM) yields the TREPR signal shown in **figure 2.3B**. The simple modification of *trans*-tetrahydroisohumulones by hydrogenation of the unsaturated side chains significantly alters the shape of the EPR signal, indicating that at least one of the radical centers originating from photolysis of *trans*-isohumulones is located in close proximity to an alkene functionality. The TREPR signal is mostly emissive due to the triplet mechanism of CIDEP, although an additional signal with two transitions can be observed on the perimeter of the spectrum: one emissive line on the low field side and a weaker absorptive line on the high field side (these transitions are marked with an asterisk in **figure 2.3B**). The fact that this signal is more strongly RPM spin-polarized (E/A) indicates that it does not occur as a primary process. It is more likely to have been generated at a later delay time following relaxation of the strong net emissive polarization from the triplet state.

Interestingly, no TREPR signal could be detected on laser photolysis at 308 nm of a solution of dihydroisohumulones (**2.3a-c**; five major constituents, individual diastereomers not assigned, **figure 2.3C**) in methylcyclohexane (ca. 10 mM). This observation provides the first direct spectroscopic evidence for the resistance of dihydroisohumulones to photolysis and confirms conclusively that radicals produced on photolysis of isohumulones and tetrahydroisohumulones originate from the α -hydroxyketone group.

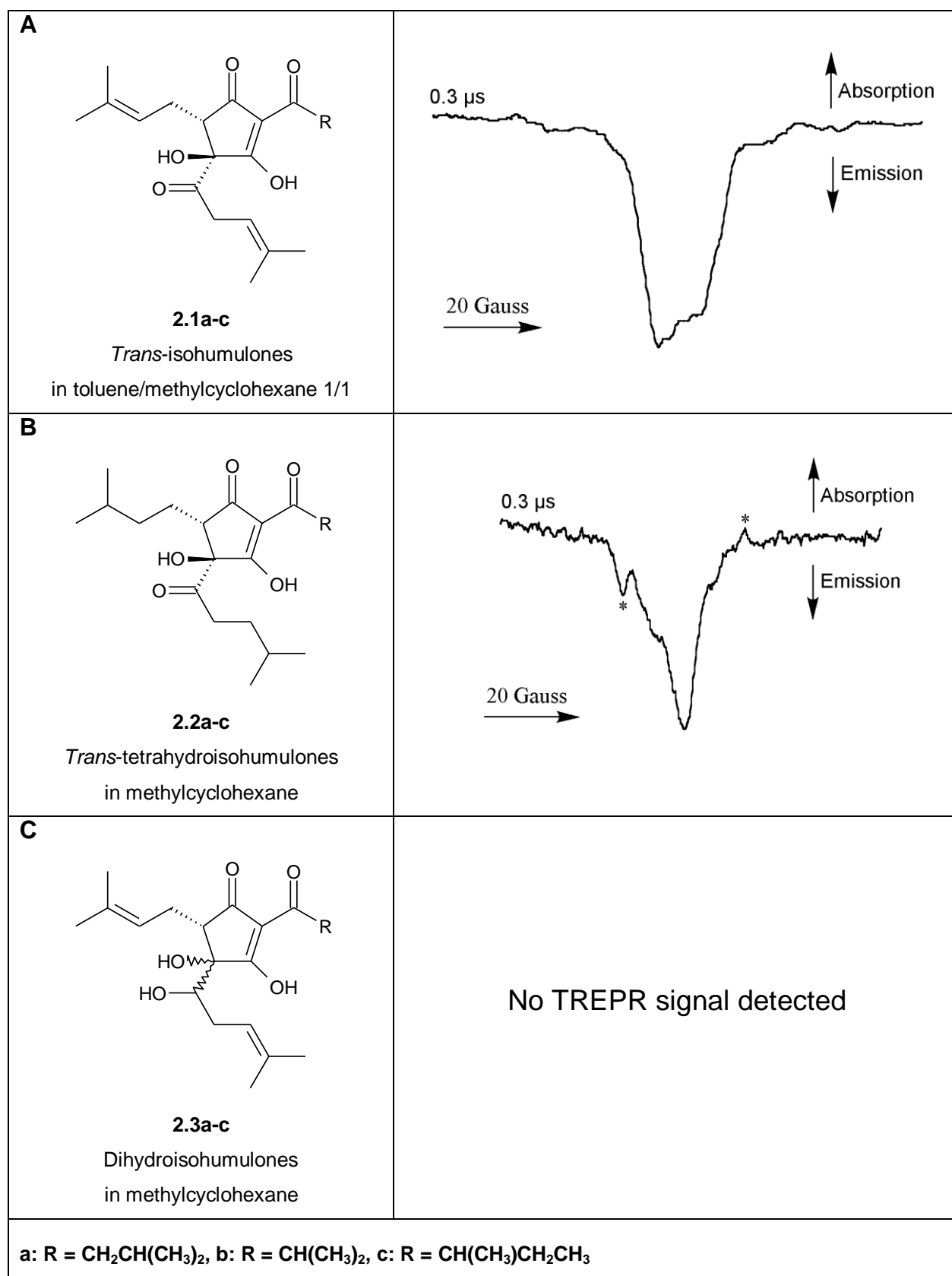
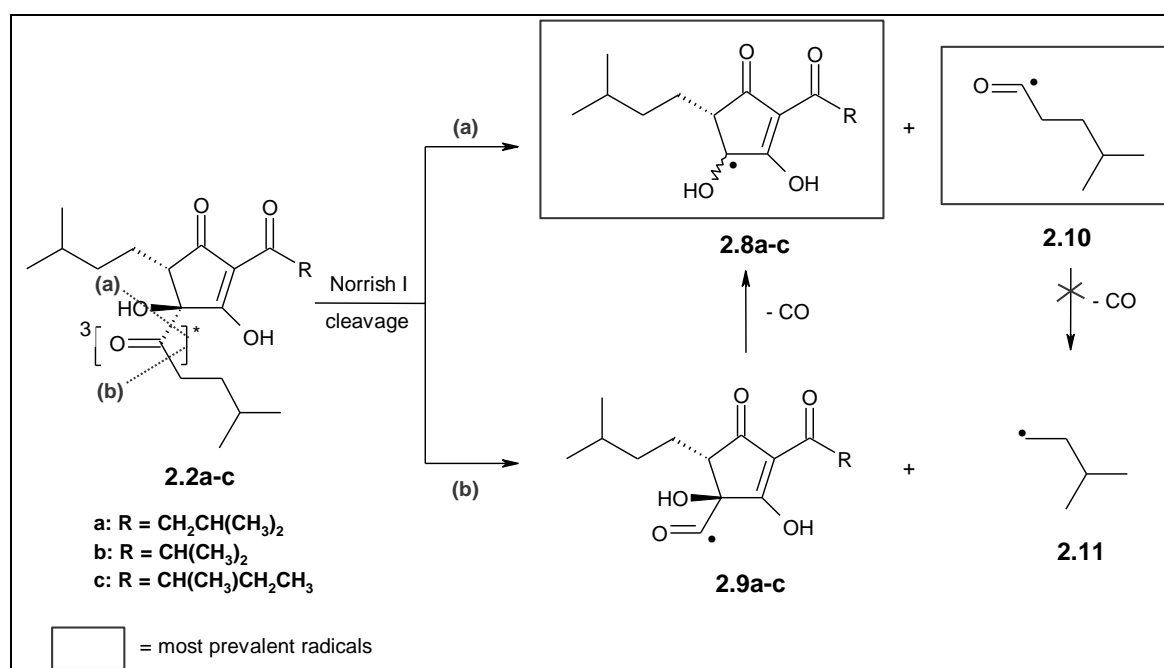


Figure 2.3

TREPR signals observed on direct irradiation (308 nm) of *trans*-isohumulones, *trans*-tetrahydroisohumulones, and dihydroisohumulones.

For *trans*-tetrahydroisohumulones (**2.2a-c**) on the other hand, cleavage via pathway (a) should be preferred, while cleavage via pathway (b) leads to a highly unstable primary 3-methylbutanoyl radical (**2.11**). In analogy, Morozova *et al.* observed only formation of an acyl-ketyl biradical on photolysis of 2-hydroxy-2,12-dimethylcyclododecanone, while an alkyl-ketyl biradical was shown to be absent.¹¹ Furthermore, decarbonylation of the 4-methylpentanoyl radical (**2.10**) does not occur on the μ s-time scale.¹² Therefore, the five-membered ring radical (**2.8a-c**) and the 4-methylpentanoyl radical (**2.10**) will be the major free radicals on Norrish Type I α -cleavage of *trans*-tetrahydroisohumulones.

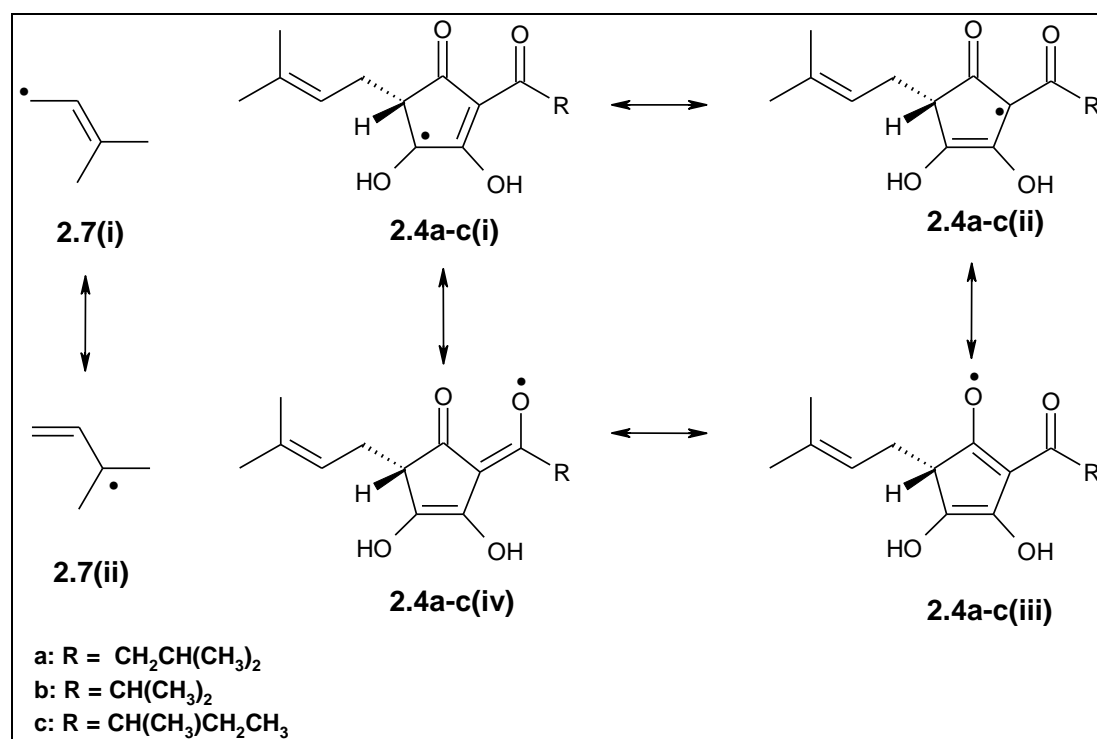


Scheme 2.2

Possible free radicals produced by irradiation of *trans*-tetrahydroisohumulones.

Differences in the predicted, most prevalent free radicals derived from Norrish Type I α -cleavage of *trans*-isohumulones and *trans*-tetrahydroisohumulones readily account for the major structural differences in the respective TREPR signals. Clearly, the TREPR signal originating from irradiation of *trans*-tetrahydroisohumulones (**Figure 2.3B**) is considerably more narrow with respect to the TREPR signal originating from irradiation of *trans*-isohumulones (**Figure 2.3A**). The changes expected in the TREPR signal on substituting a 4-methylpentanoyl radical (**2.10**) for a 3-methylbut-2-enyl radical (**2.7**) include disappearance of the hyperfine structure and appearance of a broad line with a different g-factor and no resolvable hyperfine interactions (see **Table 2.1**).

In order to give a best estimate of the hyperfine interactions, the most probable resonance forms should be taken into account (as shown in **Scheme 2.3** for radicals derived from irradiation of *trans*-isohumulones (**2.1a-c**)). The unsaturated acyl radical **2.6** should decarbonylate quickly (within approximately 100 ns) to a 3-methylbut-2-enyl radical (**2.7**). Resonance form **2.7(ii)** allows spin density on and, therefore, hyperfine interaction with the methyl protons. The hyperfine coupling constants were already reported in the literature¹² and the values, listed in **Table 2.1**, can be used in the simulation of the TREPR signal.



Scheme 2.3

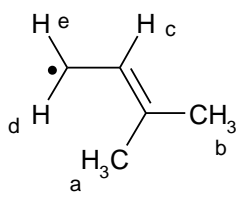
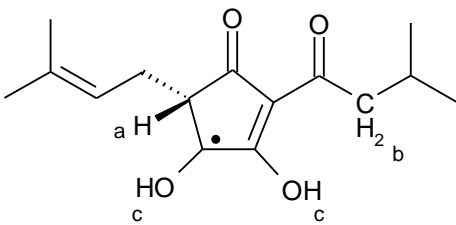
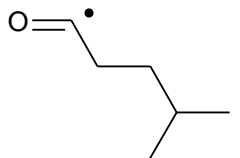
Most probable resonance forms for prevalent radicals formed on Norrish Type I α -cleavage of *trans*-isohumulones.

Radicals **2.4a-c** each have four resonance contributors (**Scheme 2.3**), although it is unlikely that they contribute equally to the overall stability. For example, resonance structures **2.4a-c(iv)** contain an exo-double bond and an electron-deficient oxygen. Structures **2.4a-c(iii)** as cyclopentadiene derivatives with an electron deficient oxygen are also probably of minor importance. Still, contributors **2.4a-c(i-iv)**, taken together, provide significant stabilization of the radical center and, because they all pull electron density away from the point of cleavage, hyperconjugation to the lone hydrogen on the ring is probably not as large as it would be for a radical without these substituents. The only radical close in structure to **2.4a-c**, for which hyperfine couplings are known, is the 3-hydroxycyclopentenyl radical,¹⁴ which has a coupling

constant of 20 Gauss for a proton in a similar position to the lone hydrogen on the ring system of **2.4a-c**. Simulation of the TREPR signal gives a coupling constant of only 8.4 Gauss for the lone ring hydrogen (**Table 2.1**). This is consistent with the expected lower spin density in our system due to delocalisation of the unpaired spin away to the other side of the five-membered ring. The hydroxyl coupling constants are also close to those reported in the literature for similar radicals.¹⁶ Presumably, they are resolved due to the use of non-aqueous solvents in our experiments; in aqueous solution, hydrogen bonding and/or exchange processes broaden them significantly.

Table 2.1

Magnetic parameters used for simulations of TREPR spectra derived from irradiated *trans*-isohumulone.

| Radical | Structure | Hyperfines (Gauss) | g-Factor | Linewidth (Gauss) |
|-----------------|---|--|----------|-------------------|
| 2.7 |  | 12.2 (3 Ha) 15.3 (3 Hb) 4.0 (1 Hc) 13.8 (1 Hd) 13.1 (1 He) | 2.0026 | 3.75 |
| 2.4a (2.8a)* |  | 8.4 (1Ha) 2.2 (2Hb) 2.9 (2Hc) | 2.0026 | 2.5 |
| 2.10 |  | No Hyperfines | 2.0008 | 7.0 |

*Radical **2.8a** (not shown) is identical to radical **2.4a**, except that the double bond in the 3-methylbut-2-enyl side chain is not present; **2.8a** has the same hyperfine coupling constants, g-factor, and linewidth as **2.4a**.

It is perhaps somewhat surprising to require hyperfine interaction on the alkyl group on the other side of the β -triketone (position b for **2.4a** in **Table 2.1**) in order to fit the spectrum. However, with delocalised radicals such as **2.4a-c**, there is certainly the possibility for hyperconjugation to the π -system leading to these small couplings. Although there are two such couplings for radical **2.4a** and only one for **2.4b** and **2.4c**, respectively, we obtain a best fit with two. This is consistent with our samples having a considerably higher mole fraction of precursors **2.1a** and **2.2a**. The possible existence of a superposition of radicals with one or two couplings at this position is most likely to be responsible for the small discrepancies in the overall line shape of the simulations, especially at the perimeter of the transitions from radicals **2.4a-c**. The possibility of hyperfine interaction with the methylene hydrogens of the 3-methylbut-2-enyl side chain can be dismissed, while the carbon atom bearing them is not directly bonded to the delocalised electrons; therefore, hyperconjugation would be very weak.

Furthermore, the relative intensities of the radicals **2.4a-c** and **2.7** appear to be quite different, but this is easily understood. The 3-methylbut-2-enyl radical has its intensity distributed over a very large number of hyperfine lines (128) that cover more than 100 Gauss. Therefore, even its most intense lines are diluted over much of the sweep width and, in fact, the least intense lines on the perimeter are undetectable and left off the total sweep in the figure. Radicals **2.4a-c**, on the other hand, have EPR signals distributed over just 18 closely spaced lines, hence this signal appears to be much more intense. In fact, the integrated intensity of each radical type is nearly the same. Small differences in the electron spin lattice relaxation time of the radicals or the decarbonylation rates of **2.5a-c** and **2.6** may be responsible for the integrations being not precisely equal. In regard to the simulation in **Figure 2.4**, it should be noted that a small amount of multiplet spin polarization (emission/absorption or E/A) has been added to this spectrum for best fit. This contribution is from the radical pair mechanism of CIDEP and we note that it is of the correct phase for a triplet precursor to the radical pair.¹⁵

For radical **2.8a-c**, the only difference being the saturation of the 3-methylbut-2-enyl side chain, identical values for the hyperfines, g-factor and linewidth can be used for the simulation. An overview of the values of the parameters used for the simulations of the TREPR spectra, together with the radicals, is given in **Table 2.1**. The simulations of the TREPR spectra, which are displayed in **Figures 2.4a** and **2.4b**, conclusively support the above findings.

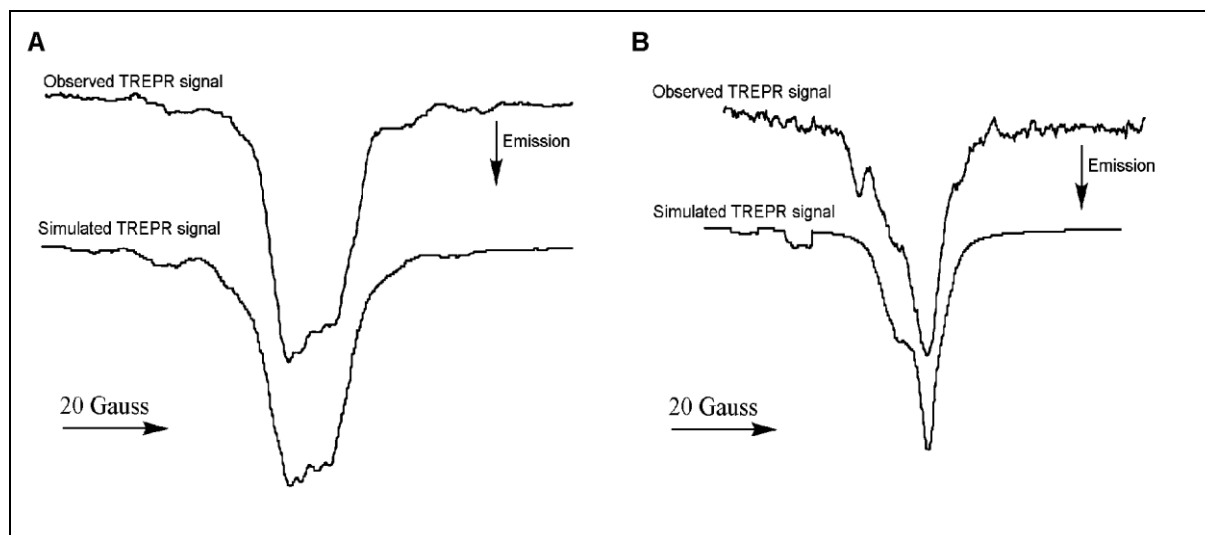


Figure 2.4

A: Observed and simulated TREPR signals on irradiation (308 nm) of *trans*-isohumulones.

B: Observed and simulated TREPR signals on irradiation (308 nm) of *trans*-tetrahydroisohumulones.

2.2.3 Further mechanistic investigations

The strong net emission exhibited in both spectra in **Figure 2.3** is generated via the triplet mechanism of CIDEP.¹⁷ Briefly, this is caused by unequal populating of the three triplet excited spin levels during intersystem crossing from the first excited singlet state. The origin of the polarization and its phase are topics worthy of further discussion, as consideration of these issues allows further mechanistic information to be obtained regarding the photophysics of the precursor molecules. In this regard, we note that the phase of TM is dependent on the sign of the zero-field splitting parameter (D) of the parent ketone. In saturated ketones, the TM is usually weak and absorptive, while ketones conjugated to unsaturated bonds exhibit stronger TM polarization that is emissive. This reversal of the TM phase is supported by reports of the sign of D for triplet states of carbonyl compounds such as cyclohexanone¹⁸ versus cyclohexenone,¹⁹ which are opposite.

The fact that dihydroisohumulones, containing only the β -triketone chromophore, do not produce a TREPR signal on laser photolysis at 308 nm can be exploited in additional mechanistical investigations. The β -triketone chromophore has a strong electronic absorption at 308 nm in both isohumulones and reduced derivatives under consideration. Reduction of the monoketone, as in dihydroisohumulones, does not alter the absorption spectrum significantly, as the weak $n-\pi^*$ transition of the monoketone must be hidden underneath the

long-wavelength edge of the strong absorption band of the β -triketone. As an example, the ϵ -value for *trans*-isohumulone (**2a**) at 308 nm in methanol is 1750 L.cm.mol⁻¹, while an ϵ -value of 15 L.cm.mol⁻¹ was found for the n- π^* -transition of a model compound, 3-hydroxy-3-methylbutan-2-one, also at 308 nm in methanol. These facts conclude that the β -triketone chromophore is the primary light-absorbing chromophore in isohumulones and reduced derivatives at 308 nm.

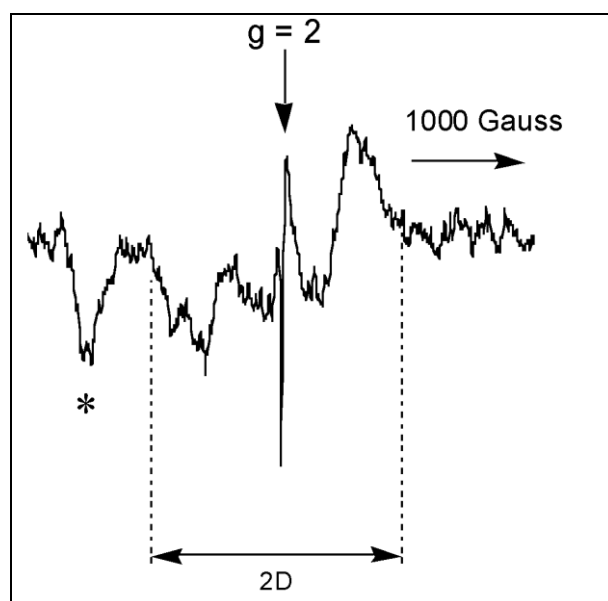


Figure 2.5

TREPR spectrum of the triplet state of frozen dihydroisohumulones.

In order to characterize the β -triketone triplet in the absence of radical formation, the triplet spectrum of a mixture of dihydroisohumulones (**2.3a-c**) was generated in frozen methylcyclohexane and observed by TREPR (**Figure 2.5**). The strongly emissive half-field transitions, indicated by an asterisk, strongly indicate that the net emissive polarization in **Figure 2.4** indeed originates from the β -triketone chromophore^c. A rough estimate of the dipolar interaction D in the triplet state can be made by measurement of the separation (in Gauss) between the outermost $\Delta m = 1$ transitions in **Figure 2.5**, indicated by dashed vertical lines. This gives $D \sim 1100 \pm 100$ Gauss or approximately 0.1 cm^{-1} , which is very consistent with a delocalized triplet such as this β -triketone.

^c The EEEAAA phase (E = emission, A = enhanced absorption) of the frozen triplet of **2.3a-c** is consistent with a structure that gives emissive TM; for example, the same pattern is observed in the randomly-oriented frozen TREPR spectrum of benzophenone.²⁰

Additional proof for the strong net emissive polarization originating from the β -triketone chromophore comes from an experiment involving photolysis of a mixture of dihydroisohumulones and 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO), a stable free radical. In the TREPR spectrum, obtained from this sample, an emissively polarized three-line spectrum of TEMPO is observed (**Figure 2.6**). No TREPR spectrum is obtained with either dihydroisohumulones alone or TEMPO alone. The emissive polarization of the TEMPO free radical observed here is a result of the radical triplet-pair mechanism (RTPM) of CIDEP, which results from the diffusive encounter and magnetic interaction between an excited triplet molecule, in this case that of the dihydroisohumulones, and a doublet state free radical. The net emission must arise from the polarized triplet state of dihydroisohumulones (**2.3a-c**) as the nitroxide does not absorb the light, nor does it have any spin polarization it can acquire on its own. Both experiments, as outlined above, allow us to conclude that the emissive polarization, observed in the TREPR spectra in **Figure 2.4**, is originally generated in the intersystem crossing process from S_1 to T_1 of the β -triketone, the primary light-absorbing chromophore in isohumulones and tetrahydroisohumulones.

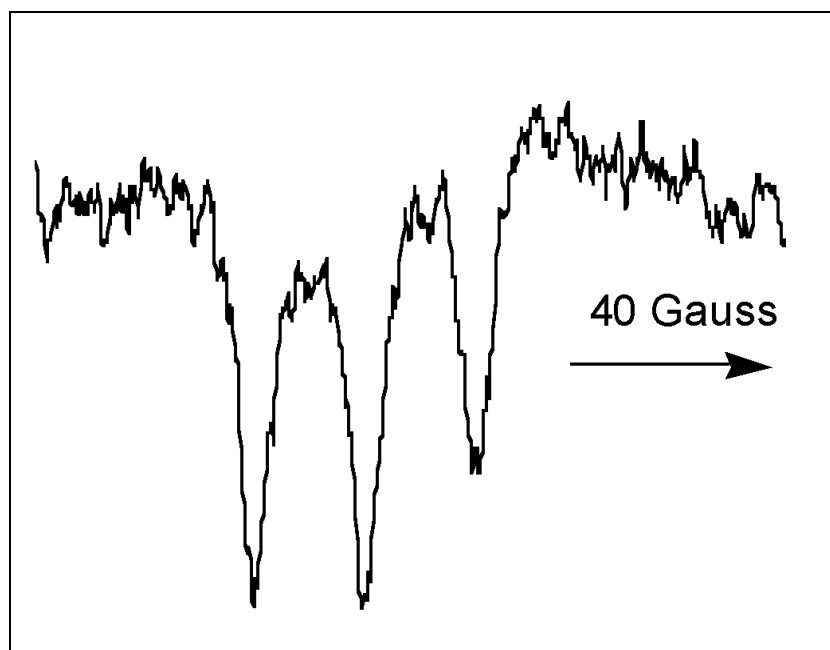
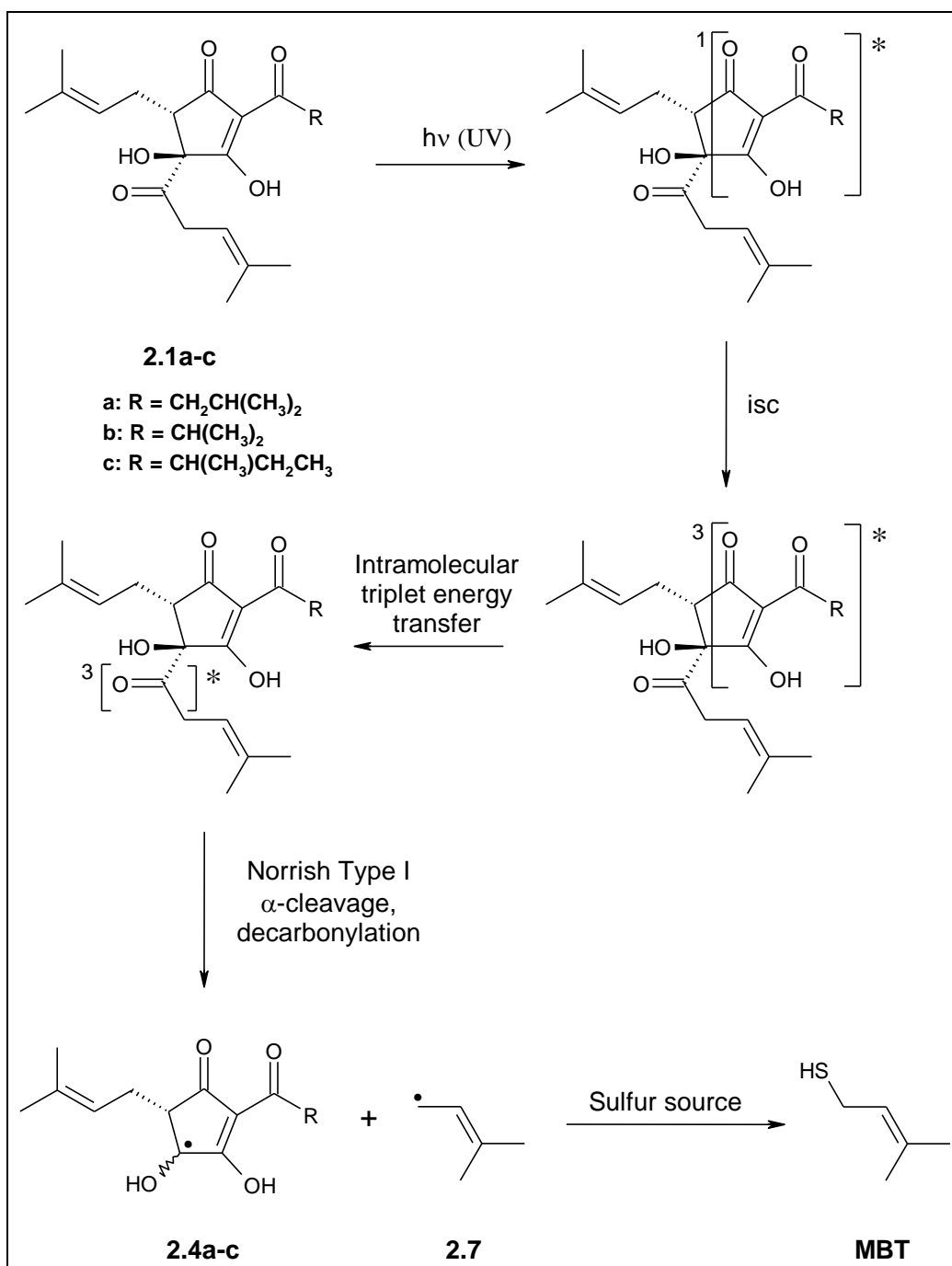


Figure 2.6
Spin polarization transfer experiment with dihydroisohumulones and 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO).

2.3 MECHANISM FOR PHOTODEGRADATION OF ISOHUMULONES AND TETRAHYDROISOHUMULONES

Combining the information collected from the TREPR experiments and simulated spectra allowed to propose a mechanism for photodegradation of isohumulones and tetrahydroisohumulones (shown for *trans*-isohumulones in **Scheme 2.4**). Absorption of UV radiation by the β -triketone chromophore leads to its first excited singlet state. After intersystem crossing (isc) to the excited triplet state, triplet energy transfer occurs to the isolated α -hydroxyketone, which in the excited state undergoes Norrish Type I α -cleavage, thereby furnishing radicals shown in **Scheme 2.1** and **Scheme 2.2**. There is good evidence supporting the assumption that spin polarization transfer accompanies triplet energy transfer,^{21,22} hence, we expect the isolated monocarbonyl triplet to show emission from the original absorbing chromophore rather than enhanced absorption from TM, which is expected if the monocarbonyl function underwent direct excitation.

Intramolecular triplet energy transfer from the β -triketone to the isolated α -hydroxyketone is noteworthy as it is expected that it should be uphill in energy by ~ 25 kJ mol⁻¹.²³ Even so, the radicals are formed from the α -hydroxyketone within a few hundred ns (the TREPR signals from **2.1a–c**, for example, show maximum intensity at approximately 400 ns after the laser-flash). For two chromophores diffusing in free solution, collisional triplet energy transfer is relatively slow ($\sim 10^5$ s⁻¹) for this large an energy difference between the states.²³ However, when the two chromophores are connected, as in this case, the triplet energy transfer rate can be much faster. Closs *et al.* measured triplet energy transfer from naphthalene to biphenyl at greater than diffusion-controlled rates (7.7×10^9 s⁻¹), when the two chromophores were connected to an alkane spacer and separated by only two C–C σ -bonds.²⁴ Although that result was for a downhill reaction (~ 17 kJ mol⁻¹), it demonstrated that covalently linking the triplet donor and acceptor can increase the intrinsic triplet energy transfer rate. It is possible that the two chromophores are, in fact, very strongly coupled in our system. Because the hydroxyl group and the acyl side chain at C-4 are oriented above and under the five-membered ring containing the β -triketone, the two chromophores are in a fair geometry for direct through-space interaction, while through-bond coupling is also expected to be very strong. Therefore, it is not unreasonable to observe this uphill energy transfer process taking place on the sub- μ s time scale.



Scheme 2.4

Mechanism for formation of 3-methylbut-2-ene-1-thiol (MBT) on direct irradiation (UV) of *trans*-isohumulones.

In order for the emissive TM polarization to be preserved during energy transfer, the transfer rate must also take place within the electron spin–lattice relaxation times of both triplets involved in the energy transfer process. This suggests that either these relaxation times are quite long or that the intersystem crossing rate from the initial excited singlet state is fairly slow. The spin lattice relaxation rate, T_1 , of the triplet electron spins depends on the tumbling rate of the triplet in solution. In fact, the magnitude of the TM-polarization also depends on

this tumbling rate. With so many alkyl appendages, tumbling will be slower than for less-substituted and more symmetric triplet states, which have electron T_1 values on the order of 1–10 ns. Therefore, a longer triplet electron spin–lattice relaxation rate than this for the triplet states of **2.1a–c** and **2.2a–c** is reasonable.

2.4 EXPERIMENTAL PART

Sample Preparation

Isohumulones are commercially available as an aqueous solution (ca. 30% w/v) of the potassium salts of *trans*–isohumulones and *cis*–isohumulones (Wigan Co., Eardiston, Near Tenbury Wells, Worcestershire, England). The isohumulones (100 mL, ca. 32 % w/v) were freed from the salts by tenfold dilution with H₂O, acidification to pH 1 with 1 N HCl, extraction with isooctane (2 x 250 mL), and removal of the solvent (yield: 30.6 g, 96%). The *trans*–isohumulones (**2.1a**: 45%; **2.1b**: 39%; **2.1c**: 16%) were accessed via selective precipitation using dicyclohexylamine (DCHA) (15.5 g) in ethyl acetate (50 mL), recovery of the DCHA–salts, recrystallization from methanol and water, redissolution in warm ethyl acetate, addition of acidified water (pH 1), phase separation and evaporation of the organic solvent (yield: 2.9 g, 9.4%).⁶

Tetrahydroisohumulones are commercially available as an aqueous solution (ca. 10% w/v) of the potassium salts (Wigan Co., Eardiston, Near Tenbury Wells, Worcestershire, England). *Trans*–tetrahydroisohumulone (**2.2a**: 42%), *trans*–tetrahydroisocohumulone (**2.2b**: 46%), and *trans*–tetrahydroisoadhumulone (**2.2c**: 12%) were separated from the *cis*–epimers according to the procedure described above for the isolation of the *trans*–isohumulones (yield: 0.4 g, 1.5%).⁶

Dihydroisohumulones (five major constituents, diastereomers not assigned individually) were a gift from Kalsec Corp., Kalamazoo, Michigan, USA.

TREPR-experiments

All spectra were recorded on a JEOL, USA, Inc. RE-1X X-Band (9.5 GHz) CW EPR spectrometer. The experimental setup is outlined above (see 2.1.2). The microwave power was varied from 0.1–20 mW. Laser excitation at 308 nm was produced using a Lambda-

Physik LPX 100i excimer laser running at a repetition rate of 60 Hz. A rectangular cavity was used with a Suprasil flat cell, 0.4 mm optical path length. The samples were bubbled with N₂ and continuously flowed to prevent sample depletion and overheating.

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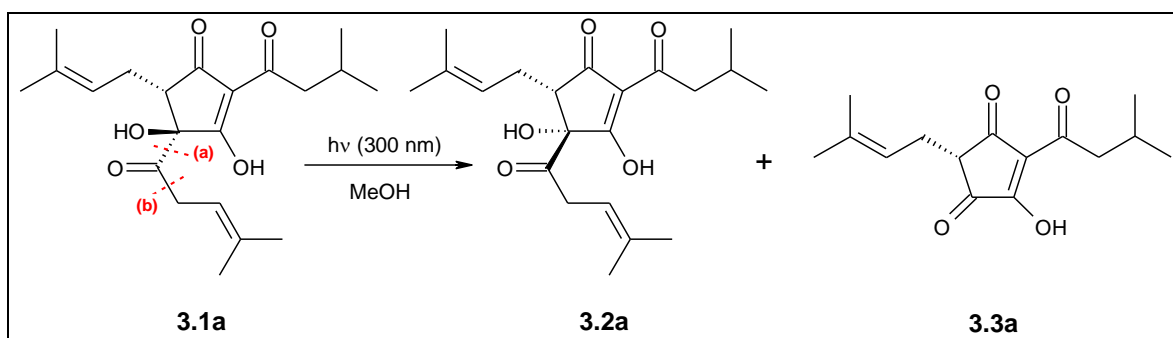
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CHAPTER 3

PHOTOREACTIVITY OF ISOHUMULONES AND REDUCED DERIVATIVES UNDER DIRECT IRRADIATION WITH UV LIGHT

3.1 INTRODUCTION

Despite the importance of isohumulones as key components in the formation of the lightstruck flavor in beer, only one investigation on the identification of photoreaction products has been reported in literature to date. Blondeel *et al.* found two major photoreaction products on photolysis of *trans*-isohumulone (**3.1a**) at 300 nm in methanol.¹ Using isocratic RP-HPLC a photoproduct eluting at the tail of the *trans*-isohumulone peak was detected and subsequently characterized as *cis*-isohumulone (**3.2a**), while an early eluting compound was identified as dehydrohumulinic acid (**3.3a**) by MS and ¹H-NMR. The formation of **3.2a** and **3.3a** was rationalized by a Norrish Type I α -cleavage from the n- π^* state of the carbonyl group in the side chain at C-4. This finding is indirectly related to the development of the lightstruck flavor, since α -cleavage of the α -hydroxyketone group furnishes a 3-methylbut-2-enyl radical (directly via route (b) or via route (a) after decarbonylation), which is a precursor of 3-methylbut-2-ene-1-thiol (MBT). On the other hand, it was found that irradiation of **3.1a** at a higher energy (254 nm) led to a very complex reaction mixture containing no major substances.



Scheme 3.1

Major photoreaction products formed on irradiation (300 nm) of *trans*-isohumulone.

The lack of information on the nature of photoreaction products derived from irradiated isohumulones led us to re-examine the photoreactivity of isohumulones in detail. Thus,

mixtures of photoreaction products derived from photostationary irradiation of isohumulones, dihydroisohumulones, and tetrahydroisohumulones, were analyzed using gradient reversed-phase HPLC-MS. Comparison of the findings should be most revealing, as hydrogenation of salient functionalities in the acyl side chain at C-4, i.e. a carbonyl group and a double bond, should correspond to clear differences in the distribution of photoreaction products, as well as in mass differences of comparable compounds.

3.2 METHOD DEVELOPMENT

3.2.1 HPLC and HPLC-MS

The HPLC separation method was adapted from a previously reported isocratic system for the separation of tetrahydroisohumulones. Using a mobile phase consisting of a mixture of acetonitrile and an aqueous buffer (48/52, v/v), Hay *et al.* were able to separate all six tetrahydroisohumulones.² The buffer solution was prepared by adjusting the pH to 2.8 with 85% phosphoric acid. Using this procedure with slightly different concentrations of organic solvent in the mobile phase, we were able to separate all six isohumulones and all six tetrahydroisohumulones, respectively, while separation of dihydroisohumulones resulted in five major peaks. We found that the nature of the octadecylsilica stationary phase was very critical for optimized peak shape and resolution. A most efficient separation was obtained with high-purity demineralized C₁₈-derivatized silica having a high carbon load and provided with efficient endcapping, such as Alltima (Alltech) and Omnisphere (Varian). Moreover, columns should be handled with extreme care, while acidic conditions (< pH 2) lead to irreversible damage of the derivatized silica, and, consequently, to loss of resolving power along with pronounced tailing. It should be noted that peak broadening is sometimes observed, especially for the later eluting adisohumulones, but the resolution was only marginally affected. Presumably, peak broadening is correlated to the existence of two or more tautomeric forms for each of the isohumulones. This isocratic method was applied for qualitative and quantitative analyses of commercially available preparations of isohumulones, dihydroisohumulones, and tetrahydroisohumulones.

For the analysis of mixtures of photoreaction products, a gradient using the same mobile phase was optimized to allow efficient separations. On switching from HPLC-DAD to HPLC-MS, some incompatibilities needed to be taken into consideration. In the aqueous part of the

mobile phase, formic acid was substituted for the non-volatile phosphoric acid, while a mixture of acetonitrile/methanol 90/10 (v/v) was substituted for acetonitrile. These modifications resulted in a drastic increase in sensitivity of the hyphenated HPLC-MS method, while the resolution was only affected to a minor extent. Electrospray ionization (ESI) both in the positive and the negative ionization modes proved necessary, while photoreaction products derived from irradiation of isohumulones and reduced derivatives showed substantial variations in ionization behavior.

3.2.2 Photostationary irradiation

In order to detect primary photoreaction products, short irradiation times - preferably in accordance with less than 5% consumption of substrate - should be used to avoid formation of secondary reaction products. While the β -tricarbonyl chromophore inherently resists photolysis, primary photoreaction products compete effectively with the original photosubstrates for radiant energy, thereby leading to formation of secondary photoreaction products and, thus, complex photoreaction mixtures. However, even at very short exposure times, a distinct number of photoreaction products was observed and the complexity of the chromatographic profiles was apparent. Therefore, it appeared reasonable to terminate the irradiation after a given time period for which the yield of photoreaction products was maximized for complete consumption of the substrates. Further exposure led to extremely complex mixtures, most likely as a result of photoinduced degradation of primary and, also, of secondary photoreaction products. Direct irradiation of the photosubstrates was performed both at 254 nm and 300 nm. The chromatographic profiles of the mixtures of photoreaction products at relatively short exposure times did not differ significantly, although, at 254 nm, extensive photodecomposition became apparent within much shorter time than observed at 300 nm. Thus, in order to decrease the complexity, irradiation at 300 nm was preferred.

Irradiations were carried out in buffered aqueous systems, methanol, and acetonitrile. Only minor differences were noticed in the distribution of photoreaction products. Thus, methanol and acetonitrile were selected as most suitable solvents, because of the high solubilities of isohumulones and reduced derivatives. Also, sample preparation and solvent removal (evaporation under N₂ or under reduced pressure) were facilitated.

3.3 DIRECT IRRADIATION OF ISOHUMULONES

Irradiation of a mixture of six isohumulones inevitably leads to very complex mixtures. Therefore, individual isohumulones, prepared from commercially available preparations, were used for the irradiations. *Trans*-isohumulones were readily available through selective precipitation with dicyclohexylamine (DCHA), while *trans*-isohumulone and *trans*-isocohumulone were accessed via semi-preparative HPLC. Although individual compounds were applied in our experiments, mixtures of photoreaction products proved to be complex, even at relatively short exposure times, as shown for the irradiation of *trans*-isohumulone at 300 nm in **Figure 3.1**.

On the other hand, HPLC-MS allowed structural assignments of a number of constituents. Based on molecular weights, UV spectral data, and retention times, photoreaction products were characterized and reaction schemes could be elaborated to account for formation of the most important constituents. An overview of identified compounds and their molecular weights is given in **Table 3.1**. It should be noted that the nature of some photoreaction products could not be revealed by lack of unambiguous MS data, hence, NMR analysis is required for full identification of all photoreaction products.

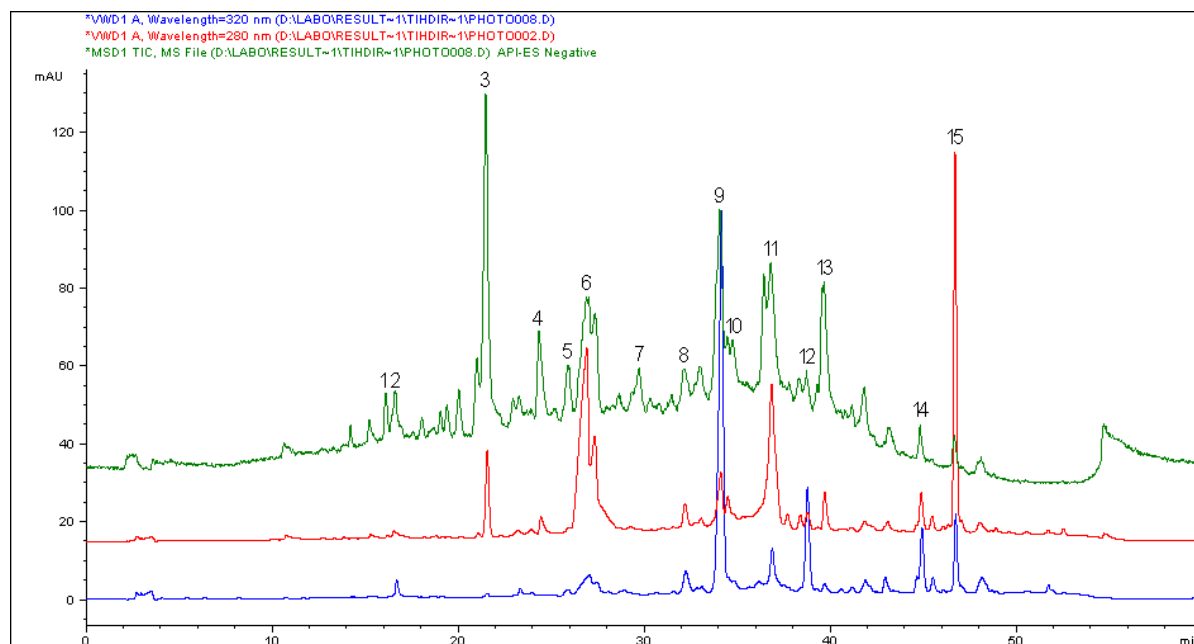


Figure 3.1

HPLC-MS analysis of a mixture of photoreaction products obtained on irradiation (300 nm) of *trans*-isohumulone (**3.1a**) for 20 min in methanol (— : UV detection at 320 nm; — : UV detection at 280 nm; — : MS in the negative ionization mode).

The characterized photoreaction products can be conveniently divided into series based on the mechanisms of formation. The results discussed below describe the mixture of photoreaction products formed on irradiation of *trans*-isohumulone at 300 nm during 20 min. Analogous results were obtained on irradiation at 254 nm, while irradiation of *trans*-isocohumulone delivered a homologous series of photoreaction products. Discrepancies from the results reported by Blondeel *et al.*,¹ are most likely due to differences in irradiation conditions and chromatographic resolution.

Table 3.1

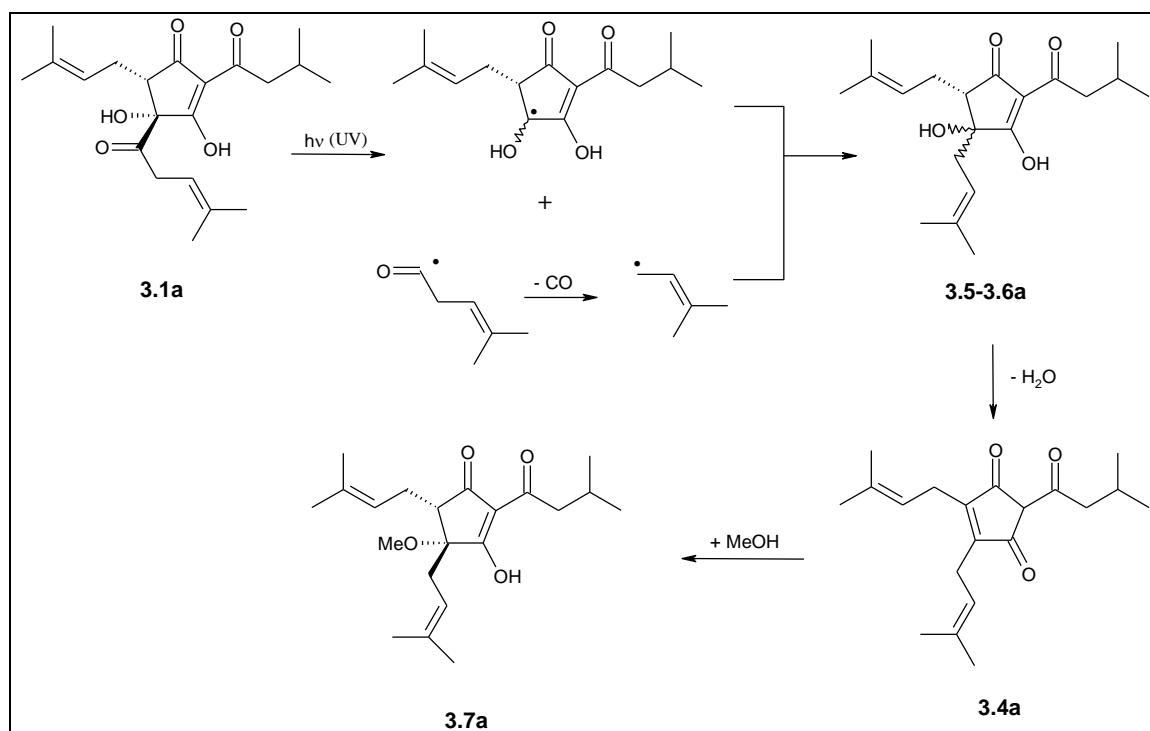
Overview of characterized compounds on HPLC-MS-analysis of the mixture of photoreaction products formed on irradiation (300 nm) of *trans*-isohumulone (**3.1a**) for 20 min.

| Peak nr. | Retention time/min | Molecular weight/amu | Δ MW/amu | Compound |
|----------|--------------------|----------------------|-----------------|----------------|
| 1 | 16.18 | 380 | 18 | 3.12a |
| 2 | 16.71 | 280 | -82 | 3.16a |
| 3 | 21.54 | 394 | 32 | 3.10a |
| 4 | 24.39 | 530 | 168 | 3.8a |
| 5 | 25.95 | 348 | -14 | 3.7a |
| 6 | 26.95 | 362 | 0 | 3.11a |
| 7 | 29.81 | 334 | -28 | 3.5a |
| 8 | 32.18 | 376 | 14 | 3.15a |
| 9 | 34.12 | 316 | -46 | 3.4a |
| 10 | 34.80 | 334 | -28 | 3.6a |
| 11 | 36.82 | 376/550 | 32/188 | 3.15a/- |
| 12 | 38.79 | 362 | 0 | - |
| 13 | 39.73 | 512 | 150 | 3.9a |
| 14 | 44.89 | 362 | 0 | 3.13a |
| 15 | 46.77 | 344 | -18 | 3.14a |

3.3.1 Photoreaction products originating from radical couplings

The major photoreaction product observed in the HPLC chromatogram recorded at 320 nm with a retention time of 34.12 min has a molecular weight of 316 amu and is formally characterized as cyclopent-2-ene-1,4-dione **3.4a** (**Scheme 3.2**). In addition to the molecular weight, which corresponds to loss of CO (- 28 amu) and H₂O (- 18 amu) from *trans*-isohumulone (**3.1a**) (362 amu), the UV spectrum (**Figure 3.2A**) (λ_{max} = 325 nm) is very informative, as it shows a pronounced bathochromic shift of about 50 nm relative to that of *trans*-isohumulone (**Figure 3.2B**) (λ_{max} = 275 nm). A cyclopent-2-ene-1,4-dione chromophore would, indeed, well accommodate the UV characteristics. The formation of this product can be rationalized as originating from splitting of the α -hydroxyketone (Norris Type I α -cleavage), followed by decarbonylation of the incipient 4-methylpent-3-enoyl

radical, recombination of the ensuing 3-methylbut-2-enyl radical and the five-membered ring ketyl radical and, finally, dehydration.



Scheme 3.2

Formation of photoreaction products on recombination of the most prevalent radicals from photolysis of *trans*-isohumulone (**3.1a**).

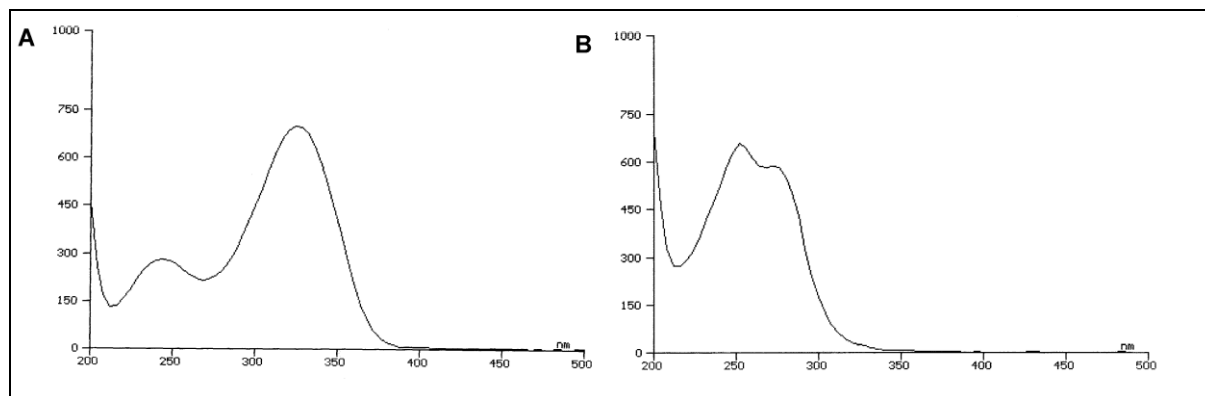


Figure 3.2

A: UV spectrum at $t_R = 34.12$ min (See **Figure 3.1**).

B: UV spectrum at $t_R = 26.95$ min (See **Figure 3.1**).

This sequence is nicely corroborated by the characterization of two photoreaction products, both with a molecular weight of 334 amu, which is consistent with the above recombination, however, in the absence of dehydration leading to the stable **3.4a**. It is very likely that the compounds represent decarbonylated *trans*- and *cis*-isohumulones **3.5a** ($t_R =$

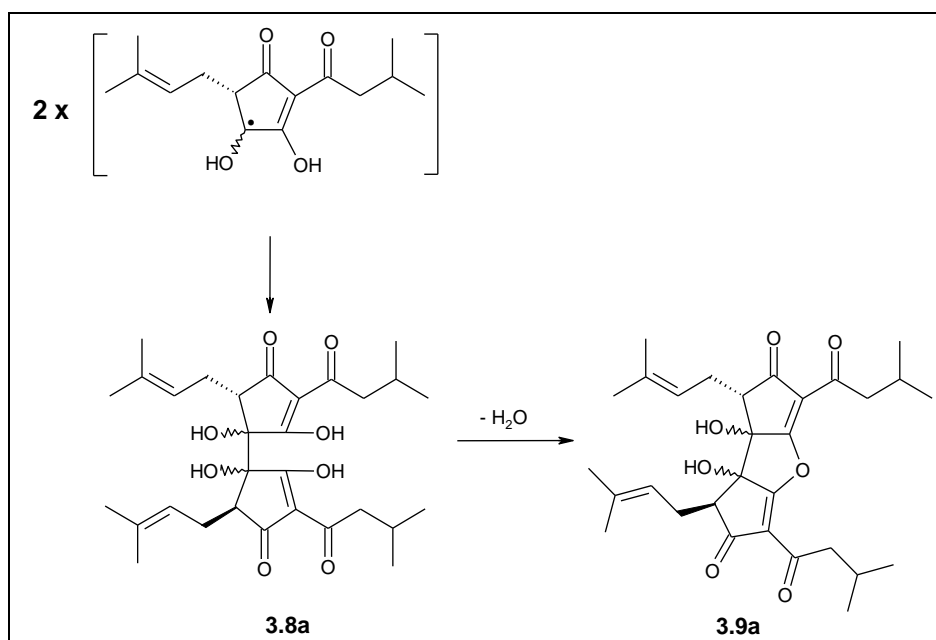
29.5 min) and **3.6a** ($t_R = 35.0$ min), respectively (the *trans*-compounds usually precede the *cis*-compounds in the HPLC conditions used for the separation). It may, furthermore, be noted that **3.5a** and **3.6a** are particularly evident at short irradiation times, which would be in agreement with the assumption that secondary excitation of the β -carbonyl chromophore in **3.5a** and **3.6a** could promote dehydration to the conjugated compound **3.4a**.

In analogy to the report by Blondeel *et al.*,¹ *cis*-isohumulone was also observed as a photoreaction product at relatively short exposure times. Formation of *cis*-isohumulone results from epimerization of the ketyl radical center and ready recombination with a 4-methylpent-3-enoyl radical. Obviously, *cis*-isohumulone is photoreactive itself, and under the reaction conditions, it escapes further control due to the complexity of the mixtures on prolonged irradiation.

Furthermore, on prolonged exposure, a further derivative of compound **3.4a** was detected, namely compound **3.7a** (MW = 348 amu; $t_R = 25.95$ min), the formation of which can be rationalized as a Michael-type addition of methanol to the enone moiety of **3.4a**. Since only one epimer has been detected, it can be expected that methanol addition occurs stereoselectively leading to *cis*-**3.7a** having the two prenyl side chains in a *trans*-configuration.

3.3.2 Photoreaction products formed by dimeric-type radical couplings

When considering the most prevalent radicals on irradiation of *trans*-isohumulone (**3.1a**), it seems likely that radical couplings between identical radicals may occur, in addition to recombination of a five-membered ketyl radical with a 3-methylbut-2-enyl radical. A peak eluting with a retention time of 24.39 min provides proof for this presumption, while the molecular weight of 530 amu clearly corresponds to a dimer arising from recombination of two five-membered ring ketyl radicals after Norrish Type I α -cleavage of the 4-methylpent-3-enoyl side chain at C-4 (Scheme 3.3).



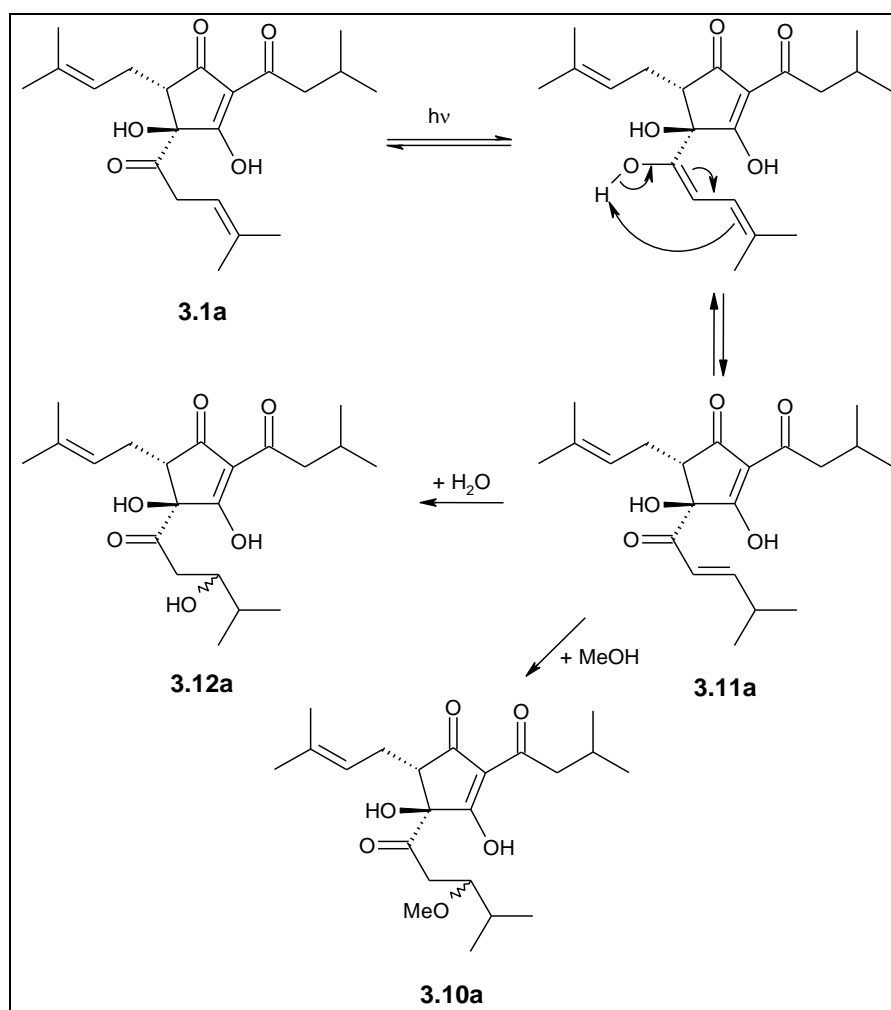
Scheme 3.3
Dimeric-type photoreaction products formed on irradiation (300 nm)
of *trans*-isohumulone (**3.1a**).

A dimer such as compound **3.8a** has not been identified previously and its existence provides further proof of a Norrish Type I α -cleavage taking place in *trans*-isohumulone and leading to a five-membered ring ketyl radical. Although three diastereomers can be formed on recombination, only one has been detected, most likely a *threo*-form. Additionally, a dehydrated dimeric compound **3.9a** (MW = 512 amu) was observed at a much longer retention time (t_R = 39.73 min), which indicates formation of a vinylogous anhydride, rather than dehydration. The UV spectrum further argues for structure **3.9a**, since the absorption characteristics of a cyclopent-2-ene-1,4-dione chromophore are absent.

3.3.3 Photoreaction products arising from addition to alloisohumulone

A major photoreaction product observed in the HPLC chromatogram recorded at 280 nm with a retention time of 21.54 min has a molecular weight of 394 amu. The increase in molecular weight of compound **3.10a** with 32 amu with respect to that of *trans*-isohumulone (**3.1a**, 362 amu) suggests incorporation of methanol. Moreover, a distinct primary photoreaction product **3.11a** (MW = 362 amu, t_R = 27.39 min) with a retention time very close to that of *trans*-isohumulone was observed, while, on prolonged irradiation, this compound was consumed. A clue to the identification of **3.11a** was provided by the MS information for compound **3.10a**. Light-induced addition of methanol to *trans*-isohumulone

(**3.1a**) can be straightforwardly rationalized by invoking initial isomerization of **3.1a** to *trans*-alloisohumulone (**3.11a**) followed by Michael-type addition of the solvent. The transposition of the double bond from a β,γ -enone to an α,β -enone is conceptually a photoisomerization occurring via photoenolization³ of the carbonyl group and concurrent tautomerization, as depicted in **Scheme 3.4**. Identification of **3.10a** is supported by the molecular weight of 380 amu found for a minor peak eluting with a retention time of 16.18 min, which is identified as hydrated *trans*-alloisohumulone (**3.12a**).



Scheme 3.4

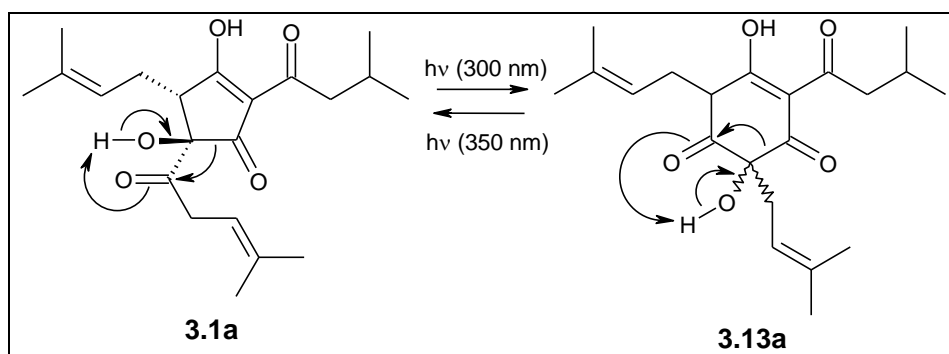
Formation of photoreaction products arising from addition to *trans*-alloisohumulone (**3.11a**) during direct irradiation (300 nm) of *trans*-isohumulone (**3.1a**).

Trans-alloisohumulone (**3.11a**) is usually formed on mild alkaline treatment of *trans*-isohumulone (**3.1a**) and, obviously, the anionic form present prior to work-up prevents Michael-type addition of water, hence isolation and identification of *trans*-alloisohumulone proves feasible.⁴ Conversely, it seems quite acceptable that the neutral conditions reigning

during photoenolization provoke Michael-type addition of methanol as a solvent and of traces of water present in methanol or derived from dehydration of intermediates. As the β -hydroxyketone **3.12a** should readily undergo a retro-aldol-type reaction, the concentration must necessarily be low. An interesting issue arising here refers to the possible intervention of photoenolization of the carbonyl group in the 4-methylpent-3-enoyl side chain at C-4 as a primary step in the photochemistry of isohumulones. This question has been raised occasionally, but the results described here provide, to our knowledge, the first indication of the existence of this pathway. Whether or not such process governs the full photoreactivity of isohumulones remains to be established.

3.3.4 Isomeric photoreaction products

The photoreaction product eluting at a retention time of 44.89 min is isomeric with *trans*-isohumulone (**3.1a**). Based on the UV spectrum and the chromatographic retention time in accordance with that of the authentic compound, it is identified as humulone (**3.13a**). While previous studies have shown that humulone can be photoisomerized to *trans*-isohumulone (**3.1a**) via a regio- and stereoselective oxa-di- π -methane rearrangement,⁵ a photo-induced reverse isomerization of *trans*-isohumulone to humulone is observed here for the first time (**Scheme 3.5**). The feasibility of this reaction derives from the presence of an oxa-di- π -methane system in *trans*-isohumulone (as well as in humulone), hence it is not surprising that a retro-oxa-di- π -methane rearrangement, in fact, occurs. An interesting, but as yet unsolved, mechanistic feature pertains to the stereoselectivity of the retro-reaction, i.e. whether or not the naturally occurring (-)-humulone is formed stereoselectively.

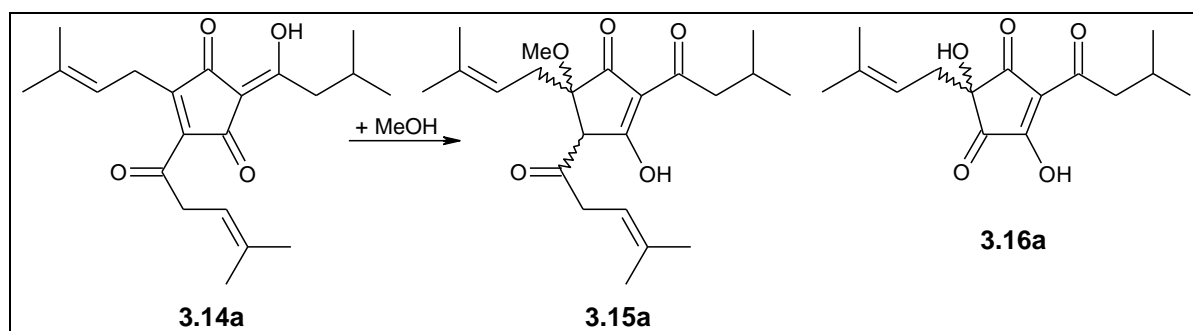


Scheme 3.5

Retro-oxa-di- π -methane rearrangement of *trans*-isohumulone (**3.1a**) to humulone (**3.13a**) and oxa-di- π -methane rearrangement of humulone to *trans*-isohumulone..

3.3.5 Other photoreaction products

In the less polar fraction of the chromatogram and at a retention time of 46.77 min, another important photoreaction product with a molecular weight of 344 amu could correspond to **3.14a** after formal dehydration of the photosubstrate (Scheme 3.6). Furthermore, Michael-type addition of the solvent to the doubly activated β -position in **3.14a** would give rise to a compound such as **3.15a** with a molecular weight of 376 amu, found for peaks eluting at 32.18 min and 36.82 min (possibly diastereomers).



Scheme 3.6

Other photoreaction products formed on irradiation (300 nm) of *trans*-isohumulone (**3.1a**).

A minor, but intriguing photoreaction product **3.16a** with a molecular weight of 280 amu was found in the more polar fraction of the chromatogram ($t_R = 16.71$ min). The low molecular weight indicates loss of the 4-methylpent-3-enyl side chain at C-4, followed by hydrogen elimination from the five-membered ring ketyl radical and concurrent oxidation. This compound is more prominently present at longer irradiation times. Remarkably, **3.16a** was found to be a major photoreaction product in the sensitized reaction of *trans*-isohumulone (see 6.2).

Obviously, a number of photoreaction products were not discussed due to lack of unambiguous MS data or insufficient arguments for characterization or identification. However, two further important photoreaction products should be mentioned, although their identities remain elusive. A compound, isomeric with **3.1a**, elutes at a retention time of 38.79 min having a characteristic UV spectrum with $\lambda_{\max} = 325$ nm. The peak eluting at 36.82 min clearly consists of two components, while the mass spectra in the negative and positive ionization modes correspond to molecular weights of 550 amu and 376 amu, respectively.

3.3.6 Conclusion

Although some photoreaction products indicate the existence of interesting minor pathways, it is clear that the majority of photoreaction products are derived from a Norrish Type I α -cleavage of the α -hydroxyketone group of the 4-methylpent-3-enoyl side chain at C-4. Moreover, the most prevalent radicals as identified by TREPR (see 2.2) readily account for the major photoreaction products via recombination of key radicals. Combination of the TREPR investigations and the detailed study on the nature of photoreaction products provides solid proof for the primary mechanism of formation of the lightstruck flavor in beer, while Norrish Type I α -cleavage of the side chain at C-4 affords precursors for 3-methylbut-2-ene-1-thiol (MBT).

3.4 DIRECT IRRADIATION OF TETRAHYDROISOHUMULONES

Preparation of *trans*-tetrahydroisohumulones (**3.17a-c**) proved much more difficult than the preparation of *trans*-isohumulones. The yield of *trans*-tetrahydroisohumulones was too low to obtain sufficient quantities of individual constituents by semi-preparative HPLC. Therefore, we preferred to carry out irradiation experiments on a mixture of *trans*-tetrahydroisohumulones, consisting of *trans*-tetrahydroisohumulone (**3.17a**, 42%), *trans*-tetrahydroisocohumulone (**3.17b**, 46%), and *trans*-tetrahydroisoadhumulone (**3.17c**, 12%).

HPLC-MS analysis of the mixture of photoreaction products, obtained on irradiation of the mixture of *trans*-tetrahydroisohumulones (**3.17a-c**) at 300 nm in methanol after 25 min, is shown in **Figure 3.3**. An overview of characterized compounds and their molecular weights is given in **Table 3.2**.

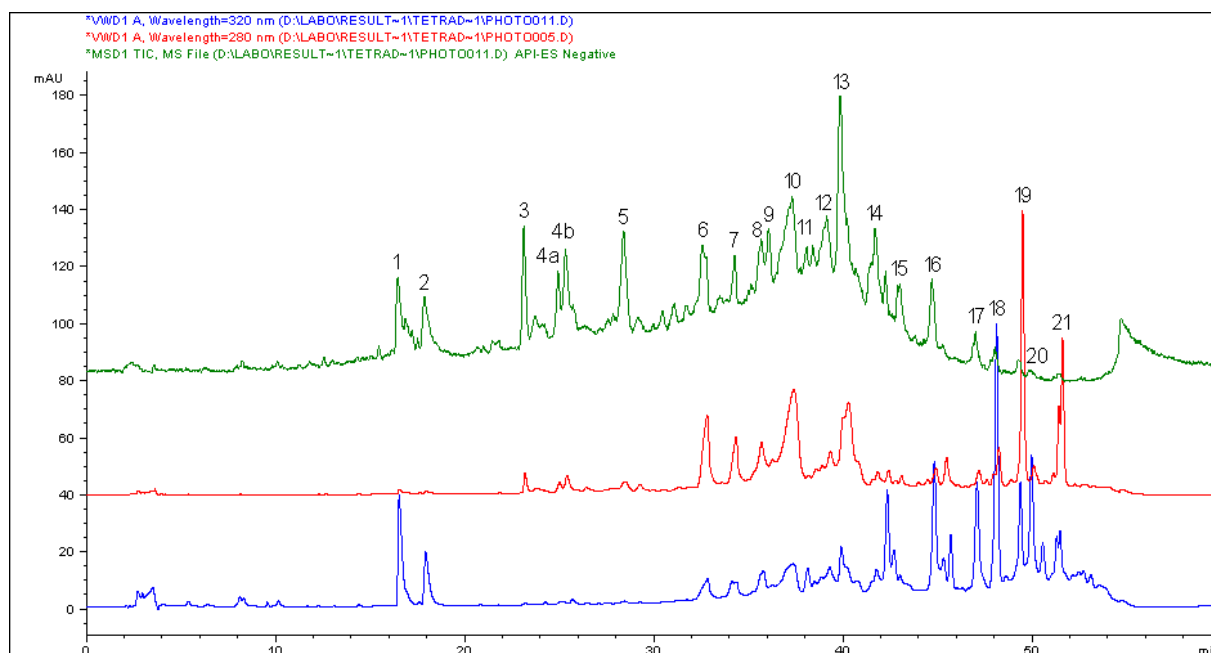


Figure 3.3

HPLC-MS analysis of the mixture of photoreaction products obtained on irradiation (300 nm) of *trans*-tetrahydroisohumulones (**3.17a-c**) in methanol after 25 min (—: UV-detection at 320 nm; —: UV-detection at 280 nm; —: MS in the negative ionization mode).

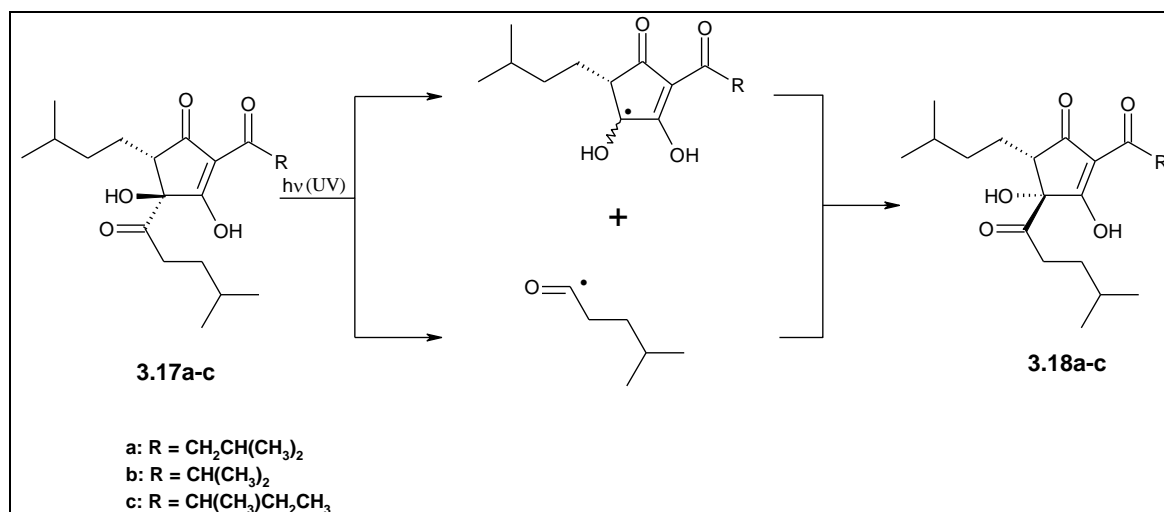
Table 3.2

Overview of characterized compounds on HPLC-MS-analysis of the mixture of photoreaction products on irradiation (300 nm) of *trans*-tetrahydroisohumulones (**3.17a-c**) for 25 min.

| Peak nr. | Retention Time/min | Molecular weight/amu | Δ MW/amu | Compound |
|----------|--------------------|----------------------|-----------------|--------------|
| 1 | 16.51 | 268 | -84 | 3.27 |
| 2 | 17.93 | 282 | -84 | 3.27 |
| 3 | 23.18 | 506 | +140/+154 | 3.20 |
| 4a | 25.00 | 520 | +154/+168 | 3.21 |
| 4b | 25.39 | 520 | +154/+168 | 3.21 |
| 5 | 28.48 | 534 | +168/+182 | 3.19 |
| 6 | 32.64 | 352 | 0 | 3.17b |
| 7 | 34.33 | 352 | 0 | 3.18b |
| 8 | 35.74 | 324 | -28 | 3.25b |
| 9 | 36.14 | 366 | 0 | 3.17a |
| 10 | 37.37 | 366 | 0 | 3.18a |
| 11 | 38.15 | 338 | -28 | 3.25a |
| 12 | 39.20 | - | - | - |
| 13 | 39.91 | 488 | +122/+136 | 3.23 |
| 14 | 41.75 | 502 | +136/+150 | 3.24 |
| 15 | 43.06 | 502 | +136/+150 | 3.24 |
| 16 | 44.77 | 516 | +150/+164 | 3.22 |
| 17 | 47.08 | - | - | - |
| 18 | 48.08 | 352 | 0 | 3.28 |
| 19 | 49.34 | 334 | -18 | 3.25b |
| 20 | 49.96 | 366 | 0 | 3.28 |
| 21 | 51.45 | 348 | -18 | 3.25a |

3.4.1 Photoreaction products originating from radical couplings

While prevalent radicals formed on direct irradiation of *trans*-tetrahydroisohumulones (**3.17a-c**) were identified by TREPR spectroscopy as a five-membered ketyl radical and a 4-methylpentanoyl radical (see 2.2), it is not surprising that, at relatively short exposure times, *cis*-tetrahydroisohumulones (**3.18a-c**) were found as primary photoproducts. Their formation can be rationalized by a Norrish Type I α -cleavage of the α -hydroxyketone group at C-4, epimerization of the five-membered ketyl radical and ready recombination with a 4-methylpentanoyl radical (Scheme 3.7). Establishment of a true photoequilibrium is hampered by further photolysis of both *cis*- and *trans*-tetrahydroisohumulones. In contrast to the low yield of *cis*-isohumulone on direct irradiation of *trans*-isohumulone, *cis*-tetrahydroisohumulones may constitute up to 25% in a mixture with *trans*-tetrahydroisohumulones.



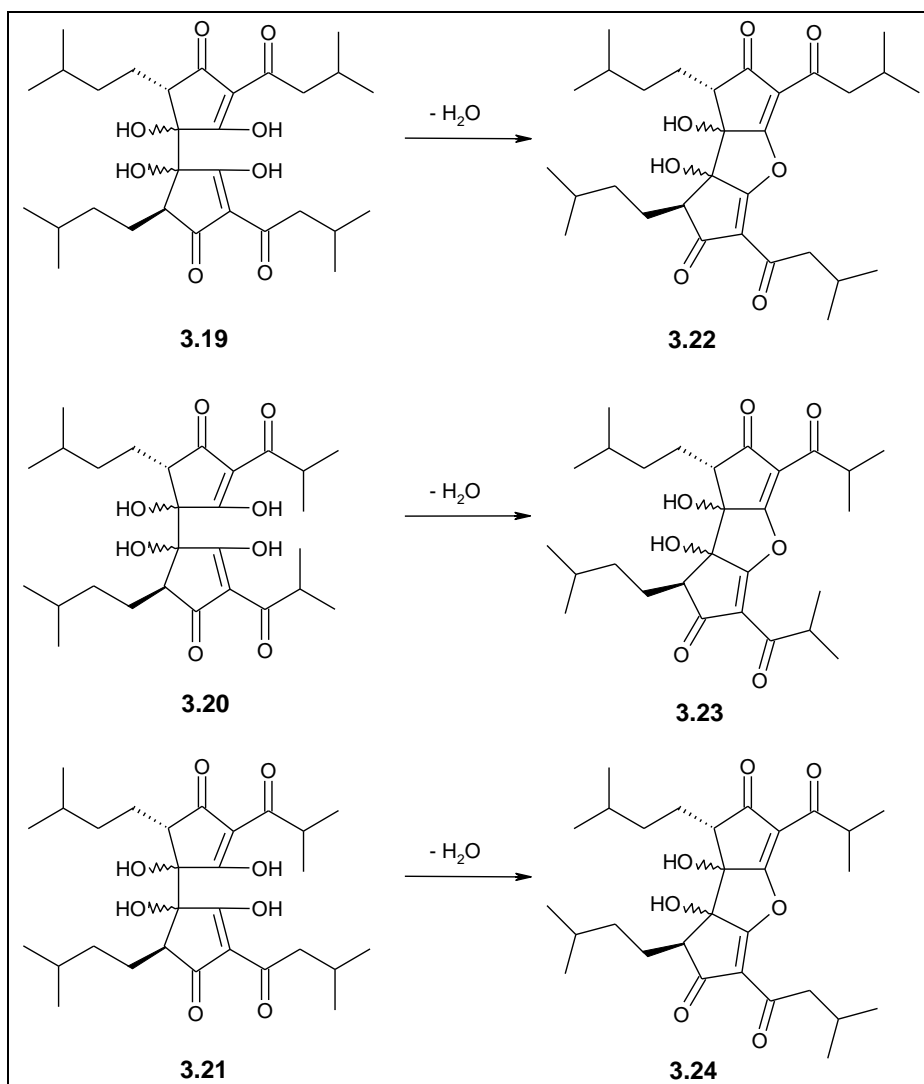
Scheme 3.7

Formation of *cis*-tetrahydroisohumulones (**3.18a-c**) on irradiation (300 nm) of *trans*-tetrahydroisohumulones (**3.17a-c**).

3.4.2 Photoreaction products formed by dimeric-type radical couplings

As observed during photolysis of *trans*-isohumulone (**3.1a**), dimeric-type compounds eluting with retention times between 20 min and 30 min were found in significant yields. Interestingly, all possible recombination products were detected corresponding to coupling of two deacylated *trans*-tetrahydroisohumulone units (**3.19**, MW = 534), two deacylated *trans*-tetrahydroisocohumulone units (**3.20**, MW = 506), and of mixed moieties (**3.21**) (Scheme

3.8). It is not known whether the original stereochemistry of the original *trans*-tetrahydroisohumulones was retained, but, since additional diastereomers were not detected, it is very likely that only *threo*-compounds were formed. Furthermore, dimers corresponding to **3.9a** have been identified as well. Thus, dehydration of **3.19-3.21** gave rise to dimeric-type compounds **3.22-3.24** with molecular weights of 516, 488 and 502 amu, respectively.



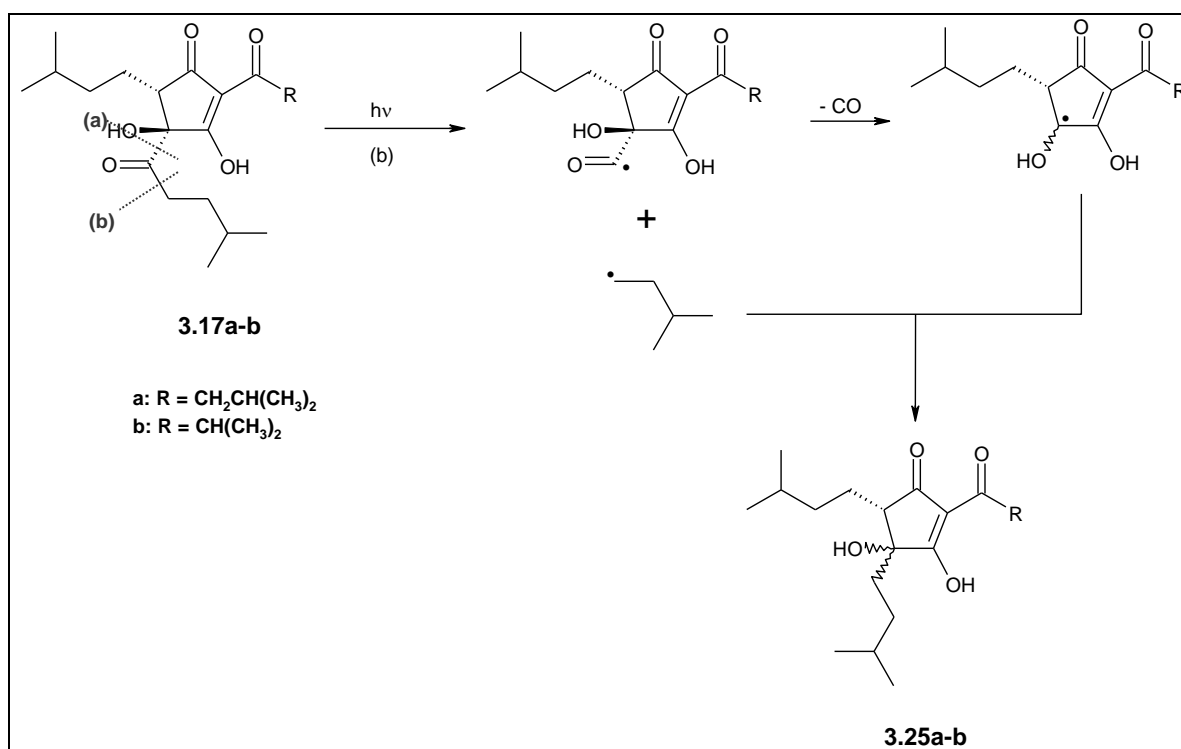
Scheme 3.8

Dimeric-type recombination products formed on irradiation (300 nm) of *trans*-tetrahydroiso-humulones (**3.17a-c**).

3.4.3 Minor photoreaction products

Identification of minor peaks with molecular weights of 338 amu ($t_R = 38.15$ min) and 324 amu ($t_R = 35.74$), respectively, is in agreement with formal loss of carbon monoxide from *trans*-tetrahydroisohumulone (**3.17a**) and *trans*-tetrahydroisocohumulone (**3.17b**) leading to

compounds **3.25a** and **3.25b**, respectively. This observation is highly revealing, since indirect proof is provided for the occurrence of a Norrish Type I α -cleavage following reaction pathway (b) as depicted in **Scheme 3.9**. Photocleavage leading to a 3-methylbutyl radical must be disadvantageous relative to pathway (a), which affords a secondary ketyl radical in addition to an acyl radical. However, decarbonylation followed by recombination would deliver decarbonylated tetrahydroisohumulones **3.25a-b**. Obviously, the alkyl radical could also recombine with a ketyl radical derived from α -cleavage via pathway (a).

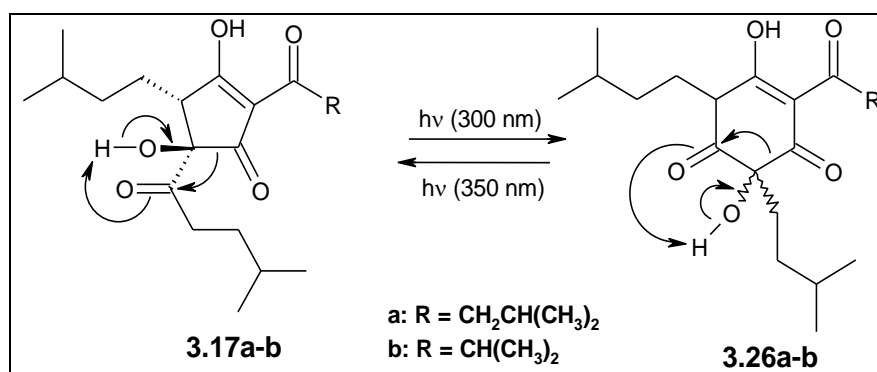


Scheme 3.9

Formation of decarbonylated tetrahydroisohumulones (**3.25a-b**) on irradiation (300 nm) of *trans*-tetrahydroisohumulones (**3.17a-b**).

3.4.4 Isomeric photoreaction products

Identification of tetrahydrohumulone (**3.26a**) and tetrahydrocophumulone (**3.26b**) with retention times of 49.96 min and 48.08 min, respectively, corroborates the existence of a retro-oxa-di- π -methane pathway from *trans*-tetrahydroisohumulone (**3.17a**) and *trans*-tetrahydroisocophumulone (**3.17b**), respectively, as was discussed for the conversion of *trans*-isohumulone (**3.1a**) to humulone (**3.13a**) (see 3.3.4) (**Scheme 3.10**).

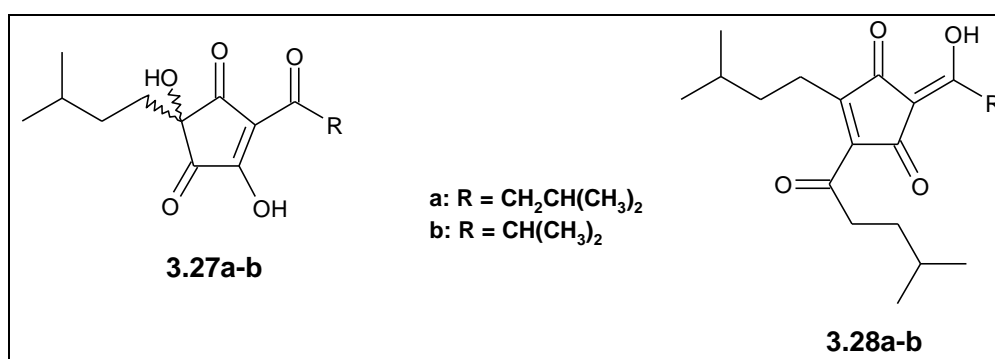


Scheme 3.10

Retro-oxa-di- π -methane rearrangement of *trans*-tetrahydroisohumulones (**3.17a-b**) to tetrahydrohumulones (**3.26a-b**) and oxa-di- π -methane rearrangement from tetrahydroisohumulones to *trans*-tetrahydroisohumulones.

3.4.5 Other photoreaction products

A further parallelism with the photoreactivity of *trans*-isohumulone (**3.1a**) was apparent by the identification of photoreaction products **3.27a-b** (t_R = 17.93 and 16,51 min; MW = 282 and 268 amu, respectively), formed by cleavage of the 4-methylpentanoyl side chain at C-4, followed by hydrogen elimination from the five-membered ketyl radical and concurrent oxidation (**Scheme 3.11**). Compounds **3.27a-b** were more prevalent at longer irradiation times.



Scheme 3.11

Other photoreaction products formed on irradiation (300 nm) of *trans*-tetrahydroisohumulones (**3.17a-b**).

Furthermore, dehydrated compounds **3.28a-b** (t_R = 51.45 and 49.34 min, respectively) were found in the less polar part of the chromatogram with molecular weights of 348 amu and 334 amu, respectively (**Scheme 3.11**), while the analogy with compound **3.14a** is obvious.

3.4.6 Relevant differences with the photolysis of isohumulones

A first pertinent observation relates to the absence of addition products similar to **3.10a** and **3.12a**, as an equivalent of *trans*-alloisohumulone (**3.11a**) does not exist, while the side chain at C-4 is saturated. Moreover, no analogs for **3.4a**, the major photoproduct on direct irradiation of *trans*-isohumulone, were observed for *trans*-tetrahydroisohumulones. Also, *trans*-tetrahydroisohumulones (**3.17a-c**) proved to be more photostable with respect to *trans*-isohumulones (**3.1a-c**) (Figure 3.4).

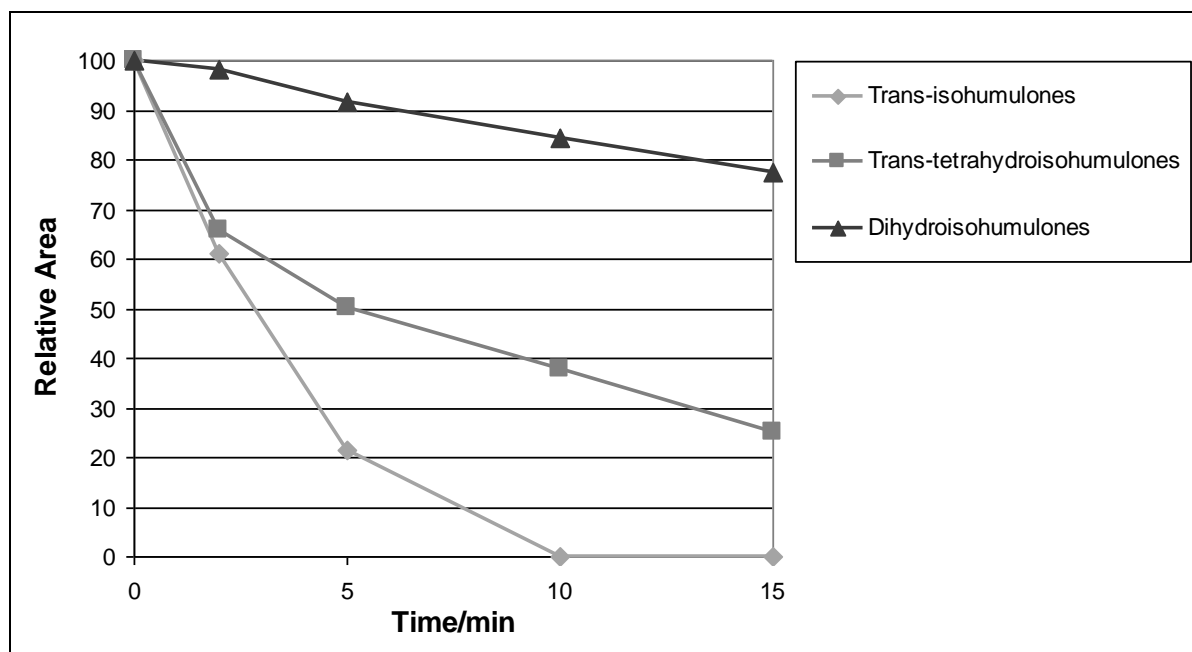


Figure 3.4

Comparison of photolysis of *trans*-isohumulones, *trans*-tetrahydroisohumulones, and dihydroisohumulones at 254 nm in MeOH (ca. 0.2 mg/mL).

As a consequence, the distribution of photoreaction products is clearly different for *trans*-isohumulones and *trans*-tetrahydroisohumulones. The quantities of minor compounds on direct irradiation of *trans*-tetrahydroisohumulones are much more pronounced with respect to direct irradiation of *trans*-isohumulones. Due to ready recombination thereby reforming the original photosubstrate, other pathways become more apparent for *trans*-tetrahydroisohumulones such as formation of dimers and dehydrated dimers, retro-oxa-di- π -methane rearrangement to tetrahydrohumulones, formal loss of the side chain at C-4 to oxidized derivatives **3.27a-b**, and dehydration of the photosubstrate. On the other hand, formation of major photoreaction products such as dimeric compounds **3.19-24** supports the

findings by TREPR thus providing solid proof that Norrish Type I α -cleavage is the most important primary photochemical event occurring in both *trans*-isohumulones and *trans*-tetrahydroisohumulones.

3.5 DIRECT IRRADIATION OF DIHYDROISOHUMULONES

Although some photodegradation is observed on direct irradiation of dihydroisohumulones at longer exposure times (**Figure 3.4**), no well-defined photoreaction products could be characterized. Remarkably, minor constituents of the mixture of dihydroisohumulones disappear significantly during irradiation, in contrast to major components, thus indicating that particular dihydroisohumulones resist photolysis. Since the carbonyl function in the side chain at C-4 has been reduced to a secondary alcohol, dihydroisohumulones cannot undergo Norrish Type I α -cleavage. Moreover, no radicals could be observed in our TREPR investigations. Therefore, it can be safely stated that dihydroisohumulones are light-stable, while photodegradation occurs to a very minor extent and only after prolonged irradiation

3.6 EXPERIMENTAL PART

Sample preparation (see 2.4)

Irradiation conditions

Trans-isohumulone (5 mg, 0.014 mmol), a mixture of *trans*-tetrahydroisohumulones (5 mg, ca. 0.014 mmol) or dihydroisohumulones, (5 mg, ca. 0.014 mmol) were irradiated in deaerated methanol (10 mL) at 254 nm or 300 nm (Rayonet photoreactor, 8 x 15 W RUL-300 nm or 254 nm lamps, The Southern New England Ultraviolet Company, Hamden, Connecticut, USA) at varying exposure times (0-60 min). Deaeration of the samples was performed by flushing with N₂ for ca. 20 min.

Chromatography

Semi-preparative Chromatography

Individual *trans*-isohumulones were isolated using a Kontron 325 HPLC system equipped with an Alltima C-18 column (250 x 10 mm, 5 μ m) and a mobile phase consisting of H₂O

(acidified to pH 2.8 with H_3PO_4) and acetonitrile in a ratio of 52/48 (v/v). UV-detection was performed at 280 nm using an ISCO single wavelength detector at a flow rate of $5 \text{ mL}\cdot\text{min}^{-1}$. Fractions containing the compounds of interest were collected, the organic solvent was removed under reduced pressure, and the remaining aqueous fraction was applied to a solid phase extraction cartridge (Varian C-18 HLB 500 mg; Varian, Harbor City, California, USA) after acidification to pH 1 (activation: 1 mL MeOH, conditioning: 1 mL H_2O (pH = 1)). Subsequent elution with MeOH (1 mL) gave the desired compounds as a residue after removal of the solvent under a gentle stream of nitrogen.

Analytical Chromatography

Isocratic analyses of *trans*-isohumulones, *trans*-tetrahydroisohumulones, and dihydroisohumulones, and gradient analyses of mixtures of photoreaction products were carried out on a Kontron system consisting of a Kontron 325 Pump and a Kontron DAD-440 Series. Samples were concentrated to ca. $2 \text{ mg}\cdot\text{mL}^{-1}$ prior to analysis. Separations (loop injections of 20 μl) were achieved on an Alltima C-18 column (250 x 4.6 mm, 5 μm) at a flow rate of $1.8 \text{ mL}\cdot\text{min}^{-1}$ (isocratic) or $1.0 \text{ mL}\cdot\text{min}^{-1}$ (gradient) using solvent A (water and formic acid, pH 2.8) and solvent B (acetonitrile/methanol 9/1 v/v). Composition of the mobile phase for isocratic analysis of *trans*-isohumulones: 52% B in A, *trans*-tetrahydroisohumulones: 62 % B in A, dihydroisohumulones: 57% B in A. Gradient profile for the analysis of mixtures of photoreaction products: 0-3 min: 28% B in A, 3-13 min: from 28 % to 55% B in A, 13-18 min: 55% B in A, 18-20 min: from 55% to 63% B in A, 20-25 min: 63% B in A, 25-31 min: from 63 % to 72% B in A, 31-48 min: from 72 % to 95% B in A, 48-50 min: 95% B in A, 50-57 min: from 95 % to 28% B in A, 57-60 min: 28% B in A.

HPLC-ESI-MS

Photoreaction mixtures were analyzed with HPLC-ESI-MS using the chromatographic conditions described above for HPLC-DAD. Samples were concentrated to $5 \text{ mg}\cdot\text{mL}^{-1}$. The chromatographic apparatus was a Hewlett Packard 1100 Series HPLC-MSD. MS conditions: electrospray ionization (ESI) both in the positive and the negative ionization modes; mass analysis with a quadrupole mass-spectrometer (API-ES: atmospheric pressure ionization - electrospray), gas temperature: 340°C , nebulizing gas pressure: 40 psi ($2.75 \times 10^5 \text{ Pa}$), drying gas flow rate of $11 \text{ L}\cdot\text{min}^{-1}$, capillary voltage: 4000 V in the positive ionization mode and

3500 V in the negative ionization mode, quadrupole temperature: 100°C, scan range: 105-600 amu and fragmentor (CID) set at 100 or 160 V in the positive ionization mode and 100 V in the negative ionization mode.

3.7 REFERENCES

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SENSITIZED IRRADIATION

CHAPTER 4

LASER-FLASH PHOTOLYSIS TRANSIENT ABSORPTION

SPECTROSCOPY OF ISOHUMULONES AND REDUCED DERIVATIVES

UNDER SENSITIZED IRRADIATION CONDITIONS

4.1 INTRODUCTION

Laser-flash photolysis coupled to emission or absorption spectroscopy is a more conventional technique - with respect to time-resolved electron paramagnetic resonance - for characterization of short-lived intermediates in photochemical processes. Flash photolysis involves production of high ‘instantaneous’ concentrations $[M^*]_0$ of short-lived intermediates using a very high-intensity pulse of radiation with very short duration. Decay of fluorescence (or phosphorescence) can be observed by monitoring the emission from the sample at right angles to the direction of the incident radiation with a spectrophotometer coupled to an oscilloscope. If only one emitting state is populated in the experiment, the fluorescence should decay exponentially according to (4.1) and the lifetime for fluorescence (τ_f) may be determined directly from the decay rate.¹

$$[M^*] = [M^*]_0 e^{-t/\tau_f} \quad (4.1)$$

Similarly, decay and formation of transients can be monitored by measuring the absorbance (at a particular wavelength) using an analyzing beam passing through the reaction cell at right angles with respect to the incident radiation. Alternatively, absorption spectra of transients can be measured as difference absorption spectra using gated detection techniques at a predetermined time interval after the initial flash. The decay of an intermediate can be monitored by recording a series of spectra at different, successive time intervals after the excitation flash. A complete spectrum of the intermediate can be constructed point by point by changing the setting of the monitoring monochromator in a series of readings at a fixed time interval after the initial flash or directly with a diode array detector (DAD).²

Obviously, the time resolution of the method is limited by the duration of the initial flash. While excited states of most organic radicals decay very rapidly (subnanoseconds to

nanoseconds for excited singlet states and submicroseconds to milliseconds for excited triplet states), the need for fast pulsed irradiation is evident. High-powered pulsed lasers are ideally suitable, while very short intense pulses of high reproducibility can be created via the technique of Q-switching or mode-locking.¹ Furthermore, lasers have the unique advantage of monochromaticity, thus the excitation wavelength can be selected to fit the compound of interest. In order to measure small absorbance changes on a nanosecond time scale, also a high-intensity pulsed analyzing light source is required to obtain good photometric signal-to-noise ratio and to reduce the effects of fluorescence and scattered laser light. A carefully designed optical system with aperture stops is used to maximize collection of analyzing light passing through the irradiation volume whilst minimizing scattered and stray light.

The set-up used in our experiments, schematically depicted in **Figure 4.1**, includes a pulsed Nd:YAG laser as an excitation source and a pulsed Xenon lamp as an analyzing beam. Rise and decay of transients can be monitored at a single wavelength using a monochromator and a photomultiplier (PM) coupled to an oscilloscope. Transient absorption spectra can be recorded directly by means of an optical multichannel analyzer (OMA).

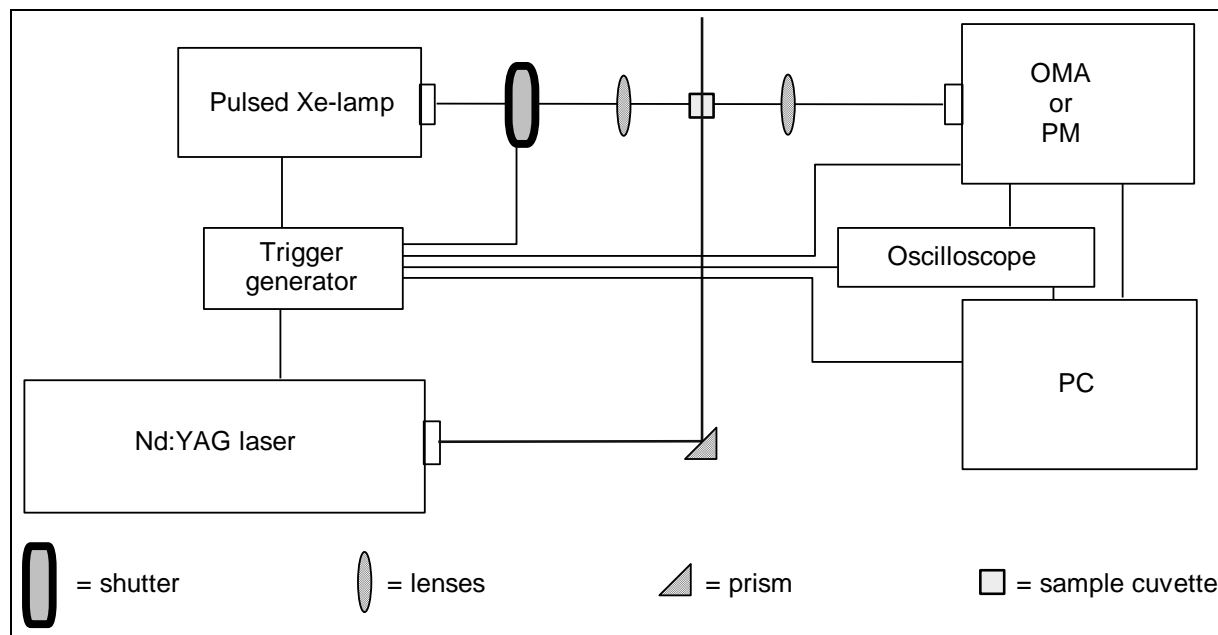


Figure 4.1

Experimental set-up of the nanosecond laser-flash photolysis spectrometer.

The timing sequence for the measurement of transient spectra is shown in **Figure 4.2**. The background noise can be reduced by repeating the measurement many times and averaging the results. The pulse from the xenon lamp lasts for 1-10 ms and the laser operates at a

frequency of 1 Hz. The detection gatewidth was set at 100 ns, while the delay time could be varied from 100 ns to over 1 ms. The transient spectrum results as the difference between the spectra measured in the presence and in the absence of the excitation pulse, respectively.

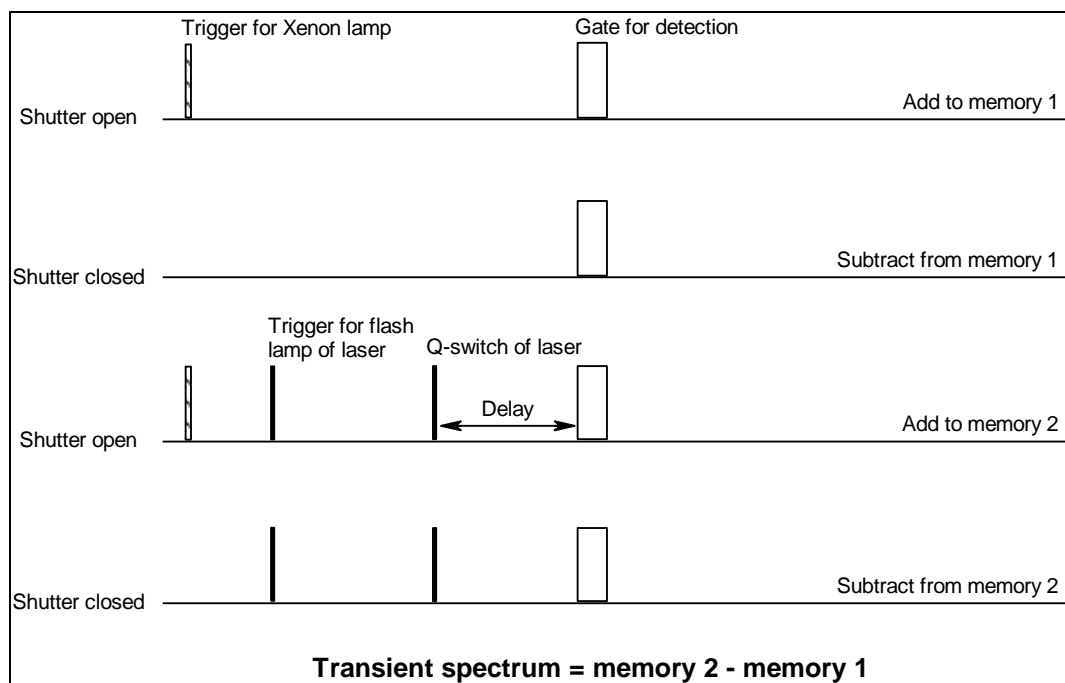
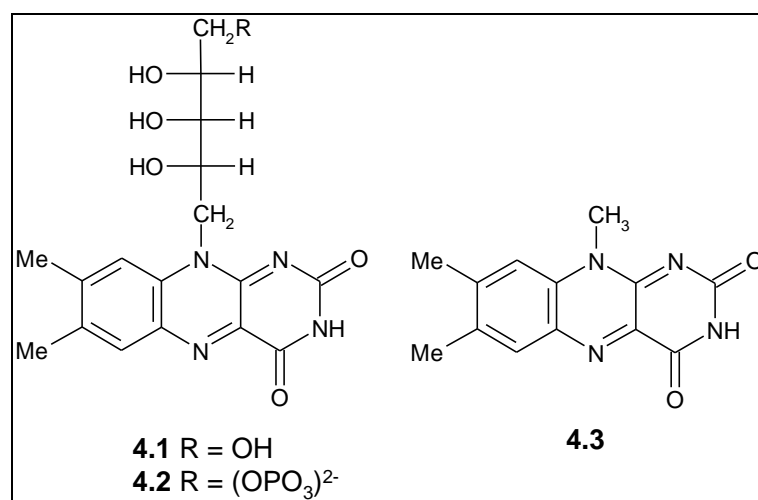


Figure 4.2

Timing sequence of the measurement of laser-flash photolysis transient absorption spectra.

4.2 TRANSIENT SIGNALS FROM IRRADIATION OF RIBOFLAVIN

The UV spectrum of riboflavin (RF) (**4.1**) (**Scheme 4.1**) shows broad absorption bands around 450 nm and 370 nm with similar molar extinction coefficients (ca. $10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$). Excitation into these absorption bands leads to identical excited singlet states due to extremely rapid internal conversion to the first excited singlet state (S_1).³ The third harmonic of the Nd:YAG laser (355 nm) is, therefore, a most suitable excitation source for riboflavin (RF) in our investigations. For an optimal response, solutions of RF were prepared with an absorbance of ca. 2 at 355 nm (ca. 250 μM). Mixtures of water/acetonitrile 50/50 (v/v) were preferred as the solubility of RF is much higher than in water or buffered systems.



Scheme 4.1

Structures of lumiflavin (**4.1**), riboflavin (**4.2**), and flavin mononucleotide (**4.3**).

4.2.1 Transient emission of riboflavin

The most straightforward experiment involves measurement of the transient emission on irradiation of RF. The emission spectrum, shown in **Figure 4.3**, was obtained after a delay time of 20 ns, while the gatewidth was set at 50 ns. The transient emission is consistent with the bright yellow fluorescence ($\lambda_{\text{max}} = 520 \text{ nm}$) resulting from the radiative transition from the first excited singlet state to the ground state reported in the literature.⁴

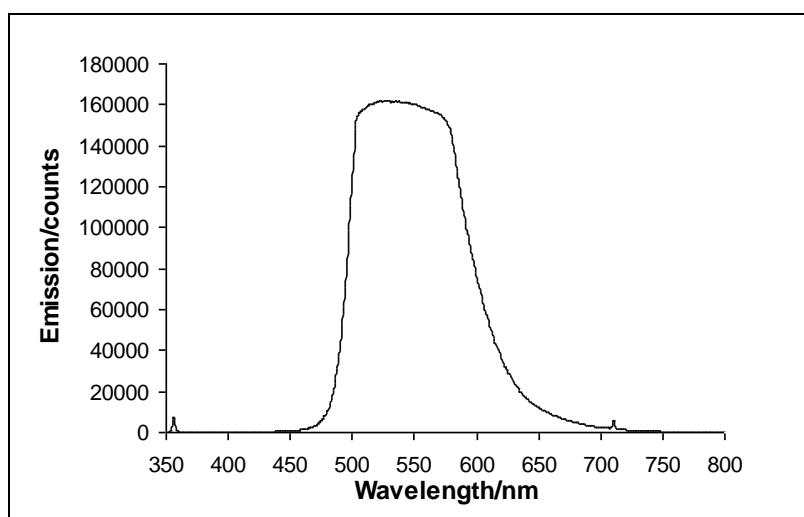


Figure 4.3

Transient emission spectrum on laser excitation (355 nm) of riboflavin (250 μM) obtained at a delay time of 20 ns with a detection gatewidth of 50 ns.

The fluorescence lifetime of RF was reported to be ca. 5 ns.⁵ Obviously, the time resolution of this experimental setup is too low to study the fluorescence decay in detail. On the other hand, it proved feasible to obtain a qualitative picture of the transient emission at short delay times. The transient emission was found to decrease very rapidly and, at a delay time of 200 ns, the transient emission signal was lost in the background noise.

4.2.2 Transient absorption of riboflavin

Laser excitation of deaerated RF (250 μM) in a 1-cm cell at 355 nm gave rise to transient absorption spectra measured at different delay times, as shown in **Figure 4.4**. The gatewidth was set at 100 ns for all delay times. The transient absorption spectrum observed at 100 ns after the laser-flash having absorption bands at 405, 530, 650 and 720 nm can be attributed to triplet-excited riboflavin (^3RF) formed via intersystem crossing from the first excited singlet state. The transient absorption spectra change significantly over the time range investigated, as a result of formation and reaction of intermediates produced during decay of ^3RF .

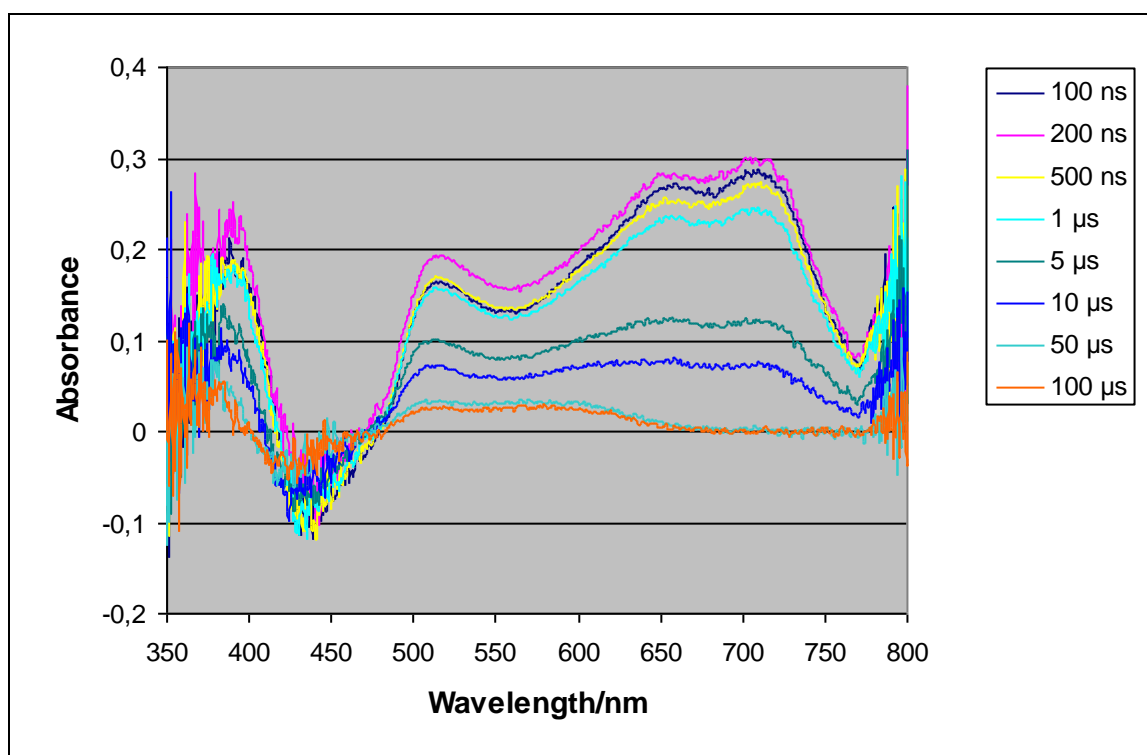


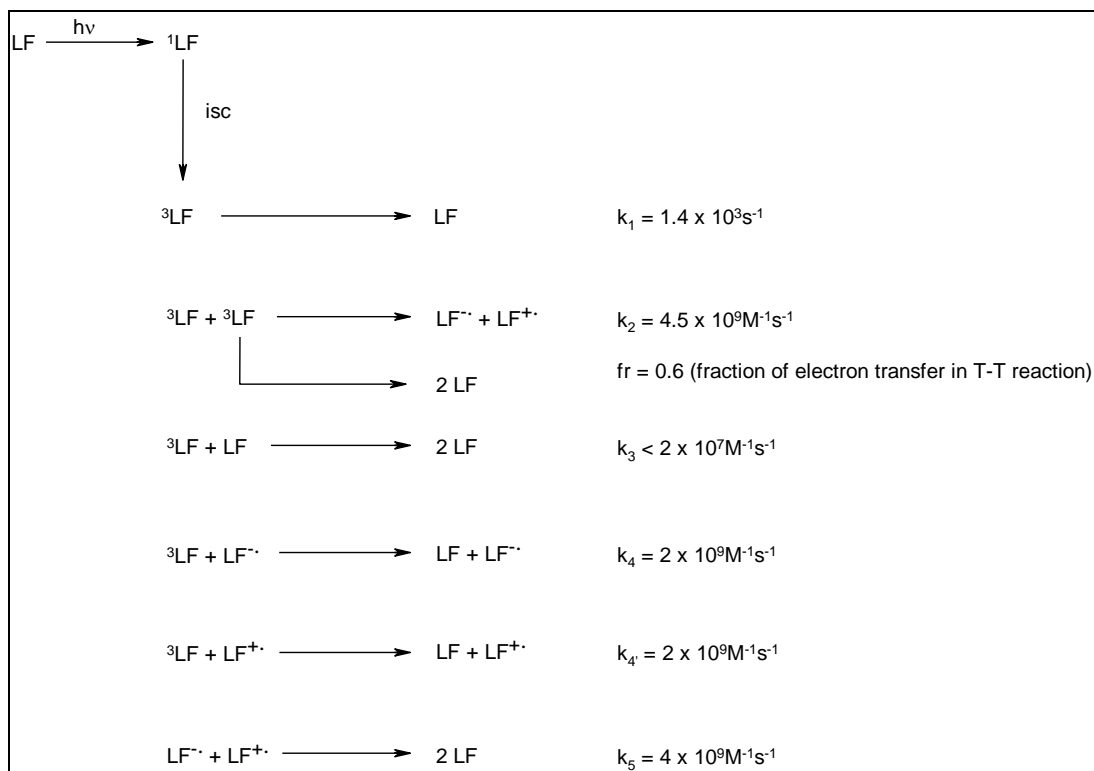
Figure 4.4

Transient absorption spectra on laser excitation (355 nm) of riboflavin (250 μM) obtained at varying delay times with a detection gatewidth of 100 ns.

At wavelengths above 700 nm, the sole absorbing species is ^3RF . The absorption band at 720 nm has completely disappeared at delay times $> 50 \mu\text{s}$, while an absorption band at 530 nm and a broad absorption band at ca. 580 nm persist much longer ($> 100 \mu\text{s}$). These two absorption bands could be attributed to the neutral one-electron reduced species of RF (**4.1**) by comparison with the difference absorption spectrum obtained on irradiation of both flavin mononucleotide (**4.2**) and lumiflavin (**4.3**) in the presence of EDTA as a one-electron reducing agent for the respective excited triplet states at a $\text{pH} < 7$.^{6,7}

Formation of the neutral semiquinone radical can be rationalized as a triplet-triplet reaction, in which electron transfer leads to both a one-electron oxidized and a one-electron reduced riboflavin radical. Depending on the pH of the solution, protonation of triplet riboflavin ($\text{pK}_a = 4.4$)⁶ or of the one-electron reduced radical ($\text{pK}_a = 8.3$)⁸ occurs. Transient absorption spectra of the one-electron oxidized species of lumiflavin, obtained by oxidative quenching of triplet lumiflavin by 1,4-benzoquinone or tetranitromethane, show an absorption band at ca. 640 nm.^{7,9} However, the transient absorption trace decreases very rapidly as a result of reaction with hydroxyl radicals or water.¹⁰ Thus, it seems reasonable that the one-electron oxidized radical of RF is decaying with a rate similar to ^3RF , and, therefore, remains hidden under the transient absorption traces for ^3RF . It should be noted that the negative absorption difference around 440 nm is due to depletion of ground-state RF.

Using low-powered flashes, Naman and Tegner¹¹ showed that first-order decay of triplet-excited lumiflavin (monitored at 720 nm) is as low as $1.5 \cdot 10^3 \text{ s}^{-1}$. High-powered excitation of solutions with varying concentrations of lumiflavin showed faster decay of triplet-excited lumiflavin in the more concentrated solutions indicating triplet-triplet annihilation. Furthermore, Yoshimura and Ohno observed significant quenching of triplet-excited lumiflavin by the radicals produced in the triplet-triplet reaction, while quenching of triplet lumiflavin by the ground state was found to be comparatively small.⁷ Our investigations with riboflavin using varying laser energies and different concentrations gave similar results as those reported for lumiflavin by Yoshimura and Ohno. An overview of the reaction mechanism for irradiation of lumiflavin, together with the rate constants, given in **Scheme 4.2**, may be adopted for RF.

**Scheme 4.2**

Reaction mechanism for dark events following excitation of lumiflavin (reproduced from Yoshimura and Ohno⁷).

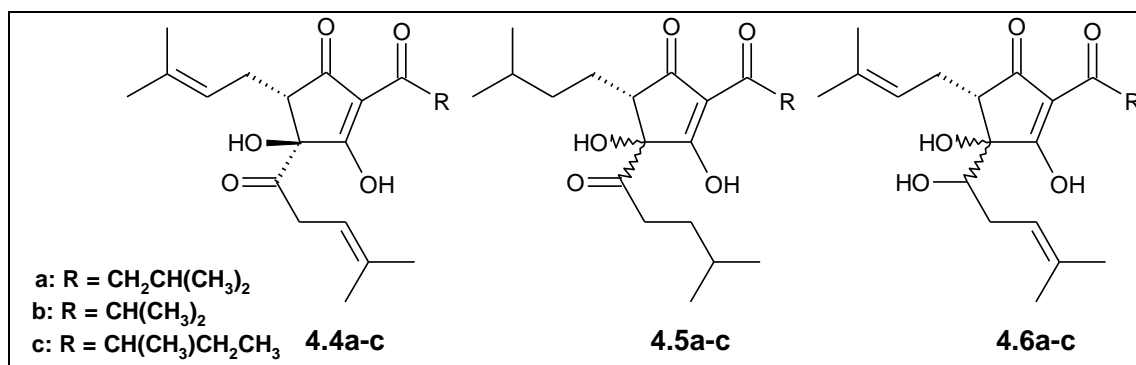
4.3 TRANSIENT ABSORPTION OF RIBOFLAVIN IN THE PRESENCE OF ISOHUMULONES AND REDUCED DERIVATIVES

4.3.1 Transient absorption of riboflavin in the presence of isohumulones

Laser excitation of deaerated RF (250 μM , acetonitrile/water, 50/50, v/v) at 355 nm in the presence of an equimolar amount of *trans*-isohumulones (**4.4a-c**) (**Scheme 4.3**) in a 1-cm cell gave rise to the transient absorption spectra at various delay times shown in **Figure 4.5**. The gatewidth was set at 100 ns for all delay times. Very similar transient absorption spectra were obtained at short delay times (200–500 ns) indicating formation of triplet-excited RF.^d However, the absorption band at 720 nm disappeared within 10 μs (at least fivefold faster with respect to RF in the absence of *trans*-isohumulones) by quenching of the excited triplet state of RF by *trans*-isohumulones. Moreover, the intense absorption bands at 520 nm and

^d The transient absorption spectrum taken at a delay time of 100 ns in **Figure 4.5** is significantly different with respect to that in **Figure 4.4** due to a minor technical problem resulting in a delay of the trigger signal with few tens of ns causing the transient spectrum to be taken few tens of ns earlier. The negative absorption differences at 355 nm and around 520 nm are due to the scattering of the laser pulse and to fluorescence, respectively. The absorption band with $\lambda_{\text{max}} = 720 \text{ nm}$ can be attributed to triplet-excited RF.

580 nm indicate rapid production of a significant concentration of the neutral semiquinone radical of RF. These observations provide solid proof for encounters between triplet-excited RF and ground-state *trans*-isohumulones followed by one-electron transfer thereby resulting in a RF-derived one-electron-reduced radical and a corresponding one-electron-oxidized radical derived from *trans*-isohumulones.



Scheme 4.3

Structures of *trans*-isohumulones (**4.4a-c**), tetrahydroisohumulones (**4.5a-c**), and dihydroisohumulones (**4.6a-c**).

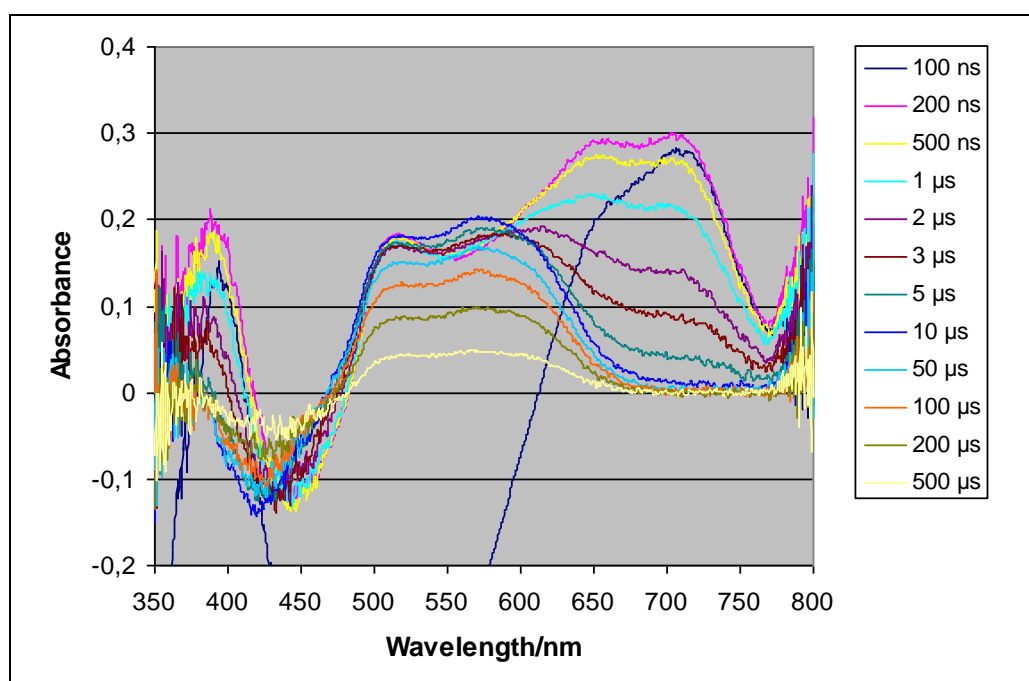


Figure 4.5

Transient absorption spectra on laser excitation (355 nm) of riboflavin (250 μM) in the presence of an equimolar amount of *trans*-isohumulones (**4.4a-c**) obtained at varying delay times with a detection gatewidth of 100 ns.

The feasibility of one-electron transfer derives from consideration of the redox potentials of the interacting species. The reduction potential of RF is 0.29 V (versus NHE) at pH 7,¹² while the excited-triplet level of RF is about 2 eV.¹³ Consequently, the redox potential of triplet-excited RF is estimated to be 1.7 V ($-0.29 \text{ V} + 2.0 \text{ V}$), hence, RF is converted to a very efficient oxidant on excitation to the triplet state. The redox potentials of important biomolecules such as amino acids, proteins, lipids, and nucleic acids are significantly lower than the redox potential of triplet-excited RF. Many investigations reported on one-electron oxidation of these substrates in the presence of RF and light.^{14,15} *Trans*-isohumulones are likewise expected to have a redox potential considerably lower than the redox potential of triplet-excited RF.

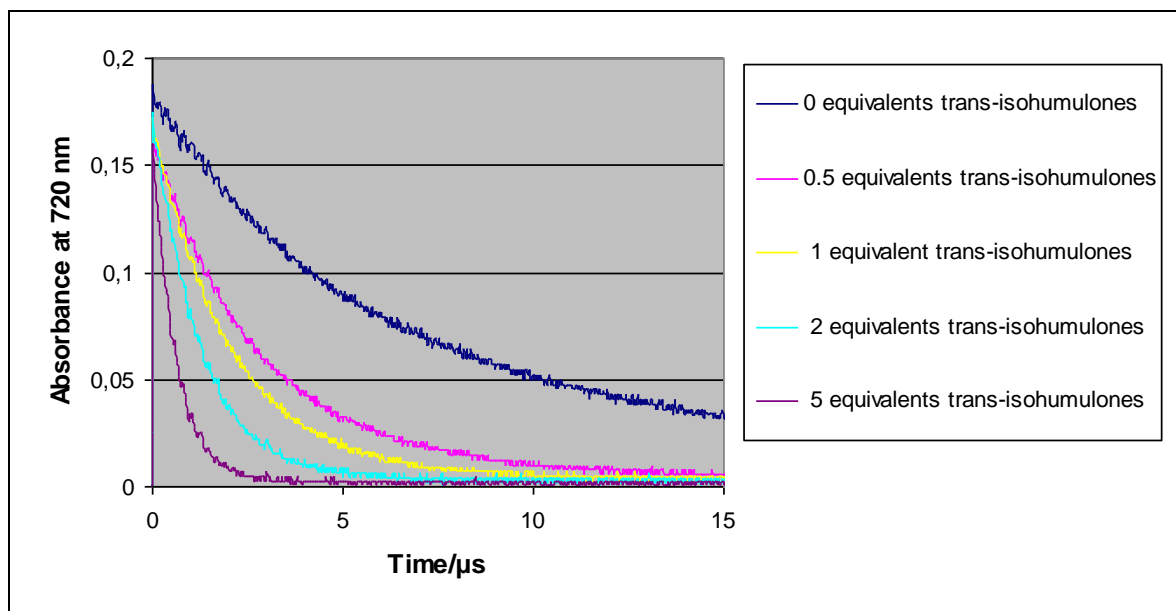


Figure 4.6

Decay of triplet-excited riboflavin monitored at 720 nm in the presence of varying concentrations of *trans*-isohumulones (**4.4a-c**).

Quenching of triplet-excited riboflavin depends on the concentration of *trans*-isohumulones, as shown in **Figure 4.6**. Even at relatively low quencher concentrations (0.5 to 5 equivalents), quenching was very significant with concurrent decrease of the excited triplet lifetimes from ca. 8 μs in the absence of *trans*-isohumulones to 400 ns in the presence of 5 equivalents of *trans*-isohumulones. The bimolecular rate constant for the reaction of *trans*-isohumulones with triplet-excited RF was determined to be $1.2 \times 10^9 \text{ M}^{-1} \cdot \text{s}^{-1}$ from the slope of the linear plot of the pseudo-first order rate constant for the decay of triplet-excited RF monitored at 720 nm against the concentration of *trans*-isohumulones (**Figure 4.7**). The

bimolecular reaction rate constant can be considered a measure of the quenching efficiency and it is related to the redox potentials of the reaction partners.

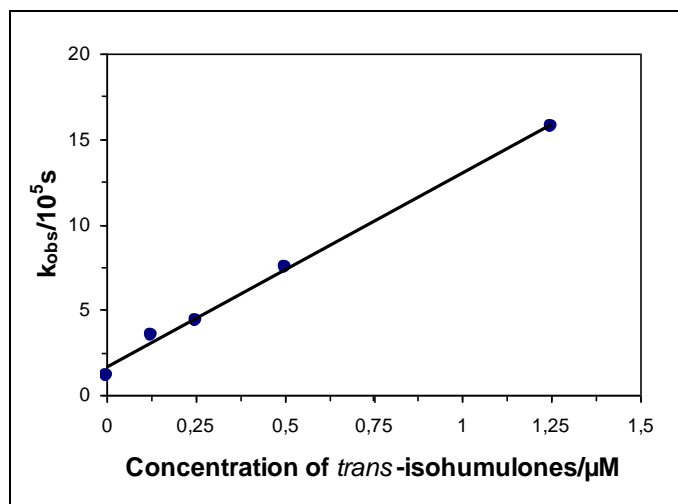


Figure 4.7

Dependence of the rate constant k_{obs} for decay of triplet-excited riboflavin observed at 720 nm as a function of varying concentrations of *trans*-isohumulones (**4.4a-c**).

4.3.2 Transient absorption of riboflavin in the presence of tetrahydroisohumulones and dihydroisohumulones

Laser excitation of deaerated riboflavin (RF) (250 μM , acetonitrile/water, 50/50, v/v) at 355 nm in the presence of 20 mole equivalents of tetrahydroisohumulones (**4.5a-c**, mixture of diastereomers) and dihydroisohumulones (**4.6a-c**, mixture of diastereomers) (**Scheme 4.3**) in a 1-cm cell gave rise to the transient absorption spectra at different delay times shown in **Figure 4.7** and **Figure 4.8**, respectively. In both instances, the transient spectra are qualitatively very similar to those observed on irradiation of RF in the presence of an equimolar amount of *trans*-isohumulones (**4.4a-c**). These observations suggest an identical reaction mechanism including formation of the semiquinone radical, derived from one-electron transfer from tetrahydroisohumulones or dihydroisohumulones in the ground state to triplet-excited RF. Obviously, increased quencher concentrations account for faster disappearance of the absorption band around 720 nm representing the rapid decay of triplet-excited RF. Although equivalent concentrations of tetrahydroisohumulones and dihydroisohumulones were added to RF, a relatively small quantitative difference regarding the decay of triplet-excited riboflavin is noted, indicating a more efficient quenching by tetrahydroisohumulones (transient signal at 720 nm disappeared after 2 μs) with respect to

dihydroisohumulones (transient signal at 720 nm disappeared after 5 μ s). This observation reflects a difference in one-electron donating properties.

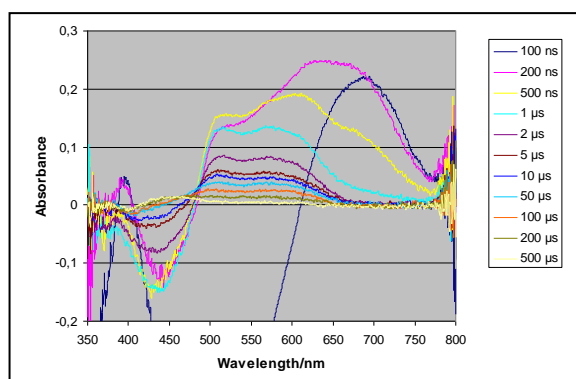


Figure 4.7

Transient absorption spectra on laser excitation (355 nm) of riboflavin (250 μ M) in the presence of 20 mole equivalents of tetrahydroisohumulones obtained at different delay times with a detection gatewidth of 100 ns.

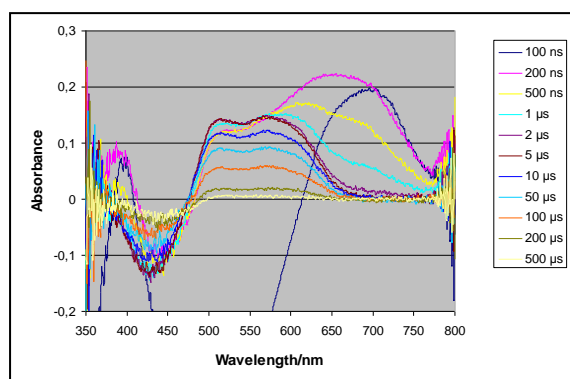


Figure 4.8

Transient absorption spectra on laser excitation (355 nm) of riboflavin (250 μ M) in the presence of 20 mole equivalents of dihydroisohumulones obtained at different delay times with a detection gatewidth of 100 ns.

4.4 EXPERIMENTAL PART

Materials

All solvents were of spectrophotometric grade (Biosolve, Valkenswaard, The Netherlands). Riboflavin was purchased from Sigma-Aldrich (Bornem, Belgium). Preparation of *trans*-isohumulones, tetrahydroisohumulones, and dihydroisohumulones was performed as described in the experimental part of Chapter 2 (see 2.4).

Nanosecond laser photolysis transient absorption and emission

A Spectra Physics GCR 3 pulsed Nd:YAG laser was used as an excitation source. The fundamental wavelength of 1064 nm (10 Hz, 8 ns) was frequency-tripled to 355 nm using a SHG-2 Harmonic Generator. A non-focused laser pulse of 4-16 mJ was applied to excite the sample. Using a pulsed 450 W xenon lamp (Müller Elektronik-Optik LAX 1450 with SVX 1450 current supply and MSP 05 pulsing unit) an intense and narrow (1-10 ms) pulse of the analytical light source was generated. The analytical beam was then shaped by means of a 1 x

2 mm slit and the excitation beam, perpendicular to the analytical beam, had a cross section of 2 x 10 mm. A power meter was arranged behind the cell. For transient absorption experiments non-focused laser pulses of 4-16 mW were used to excite the sample. In the transient emission experiments, the analytical beam was not applied.

By means of the OMA II system (EG&G Instruments) spectra were analyzed simultaneously over the wavelength region of 350-750 nm. The delay between the laser-flash and the recording of the spectra was varied using a high-voltage pulse generator (model 1302 Fast Pulser with 5-10-20 ns gate) and an intensified silicon photodiode array detector (model 1421) (increment delay from 20 ns to 1 ms). The gatewidth in the experiments varied from 20 ns to 100 ns (supplied by the 1302 Pulse Generator). The detector consisted of a microchannel plate (MCP) intensifier coupled by fibre optics to a silicon photodiode array (SPD). The apparatus was controlled by an OMA console, which provided the output trigger pulses, a 1463 detector scan controller, and the signal processing equipment.

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CHAPTER 5

STEADY-STATE AND TIME-RESOLVED ELECTRON PARAMAGNETIC RESONANCE OF ISOHUMULONES AND REDUCED DERIVATIVES UNDER SENSITIZED IRRADIATION CONDITIONS

5.1 INTRODUCTION

Time-resolved electron paramagnetic resonance (TREPR) has been proven successful in elucidating intricate details of the primary mechanism of formation of the lightstruck flavor in beer (**Chapter 2**). It was, therefore, straightforward to apply the powerful technique to investigate the role of isohumulones along with reduced derivatives in the formation of the lightstruck flavor in beer on sensitized irradiation. In analogy with the experiments described previously (**Chapter 4**), the third harmonic of the Nd:YAG laser (at 355 nm) was applied as a most suitable excitation source for riboflavin (RF) with respect to its stability, high power, convenience, and ready availability. Although experiments using well-known model systems produced a very good signal-to-noise response, no TREPR signals could be observed on irradiation at 355 nm of solutions containing RF (0.24 mM) on a time scale between 100 ns and 8 μ s. As this appeared to be due to lack of sensitivity, application of various solvents and buffers led to an optimized concentration of 0.8 mM RF in acetonitrile/water 1/1 (v/v) (ca. 15 mg in 50 mL). On the other hand, the concentration of the radical anion of RF can be increased, when EDTA is added as a one-electron reducing agent of triplet-excited RF. Even, taking into account these additional precautions, no TREPR signal was apparent. Conversely, the higher sensitivity of steady-state experiments could be exploited to detect a steady-state concentration of RF-derived radicals under continuous irradiation, although time resolution and mechanistical information are lost.

5.2 STEADY-STATE ELECTRON PARAMAGNETIC RESONANCE (SSEPR)

A 1-kW high-pressure mercury arc, exhibiting a broad emission in the UV and the visible wavelength regions, was preferred over a laser as the excitation source (set at 400 W) in the steady-state electron paramagnetic resonance (SSEPR) experiments. Also, the resonator was

altered and a cylindrical sample cell (id: 1 mm) was used instead of the 0.4 mm cell with flat windows. As a control, and in the absence of light, a 1 mM solution of TEMPO (2,2,6,6-tetramethylpiperidine-*N*-oxyl (**5.1**), a stable nitroxyl radical) resulted in a very intense three-line signal ($a_N = 16.7$ G), while also ^{13}C -satellites could readily be observed (marked with asterisks) (**Figure 5.1**).

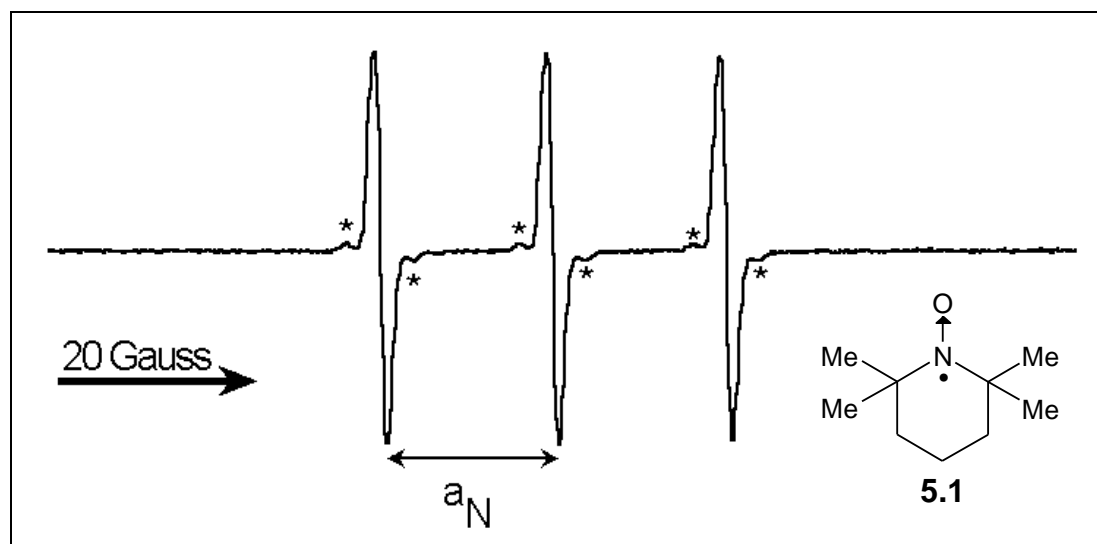


Figure 5.1

SSEPR-spectrum of a 1 mM solution of 2,2,6,6-tetramethylpiperidine-*N*-oxyl (TEMPO) (**5.1**) in water.

Under identical conditions, but in the presence of light, a mixture of 0.8 mM RF and 8 mM EDTA (acetonitrile/water, 1/1, v/v) was investigated by SSEPR. While no signal was observed initially, a small amount (ca. 2 mL) of the TEMPO solution (1 mM in water) was added in order to control the response of the instrument. At first, a signal identical to that in **Figure 5.1** was observed, but the intensity of the signal decreased rapidly over few minutes, while no other signal became apparent. In order to characterize the interacting species leading to quenching of the TEMPO signal, four model systems were evaluated with SSEPR under irradiation: (a) 1 mM TEMPO, (b) 0.7 mM RF and 1 mM TEMPO, (c) 0.7 mM RF, 7 mM EDTA, and 1 mM TEMPO, (d) 7 mM EDTA and 1 mM TEMPO. The signal originating from TEMPO did not change significantly during irradiation (> 1 h) in solutions (a), (b), and (d,) indicating the photostability of TEMPO on direct irradiation. However, in the presence of both RF and EDTA (solution (c)) the EPR signal originating from TEMPO was efficiently quenched. Furthermore, the rate of disappearance was found to be concentration-dependent. For a sample containing 0.1 mM TEMPO, 0.7 mM RF, and 7 mM EDTA the TEMPO signal showed complete disappearance after an irradiation period of only 3 min, resulting in a

marked decrease in intensity of the signal even during time needed to take 1 scan (1 min) (**Figure 5.2**).

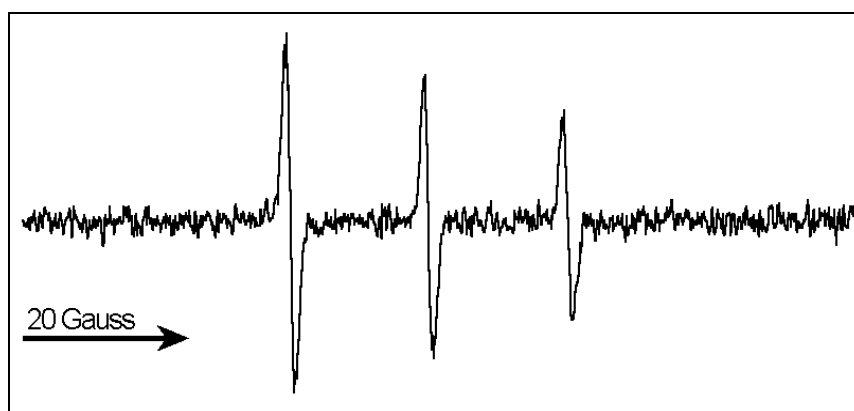


Figure 5.2

SSEPR spectrum of a solution containing 0.1 mM 2,2,6,6-tetramethylpiperidine-*N*-oxyl (TEMPO), 0.7 mM riboflavin, and 7 mM EDTA under steady-state irradiation (recording time: 1 min).

The main reactions of nitroxide radicals resulting in loss of paramagnetism are oxidation, reduction, and free-radical recombination.¹ Our results indicate that free-radical recombination is the major factor contributing to the quenching of the TEMPO-derived EPR signal. Only when RF is present, radicals can be formed on irradiation. In the absence of EDTA, the steady-state concentration of RF-derived radicals is low, while addition of an efficient one-electron donor results in significantly higher concentrations of the semiquinone radical and the corresponding cation radical formed via one-electron transfer from EDTA to triplet-excited RF. Only a sufficient concentration of free radicals leads to rapid quenching of TEMPO.

Moreover, by changing the field modulation width (FMW) from 0.1 G to 5 G, a weak and broad signal became apparent, when the signal of TEMPO had disappeared completely (**Figure 5.3**). The signal in the center of the spectrum (marked with asterisks) is due to a background signal resulting from the anisotropy of the cylindrical quartz sample cell. The broad signal corresponds to a flavin semiquinone radical. The width of the signal results to a great extent from unresolved ¹⁴N-hyperfine couplings that occur in view of appreciable spin density on *N*-5 and *N*-10 of the flavin ring. Additionally, substantial couplings are feasible to the methyl protons at C-8 and, for the neutral radical, to the proton at *N*-5.^{2,3} The neutral and anionic flavin radicals can be readily distinguished because loss of a hyperfine coupling to the

proton at *N*-5 in the anion results in a characteristic narrowing of the EPR linewidth from 18-19 G for the neutral radical to ca. 15 G for the anionic radical.⁴ The somewhat broader linewidth of 21.0 G may be caused by interaction with the cation radical of EDTA.⁵

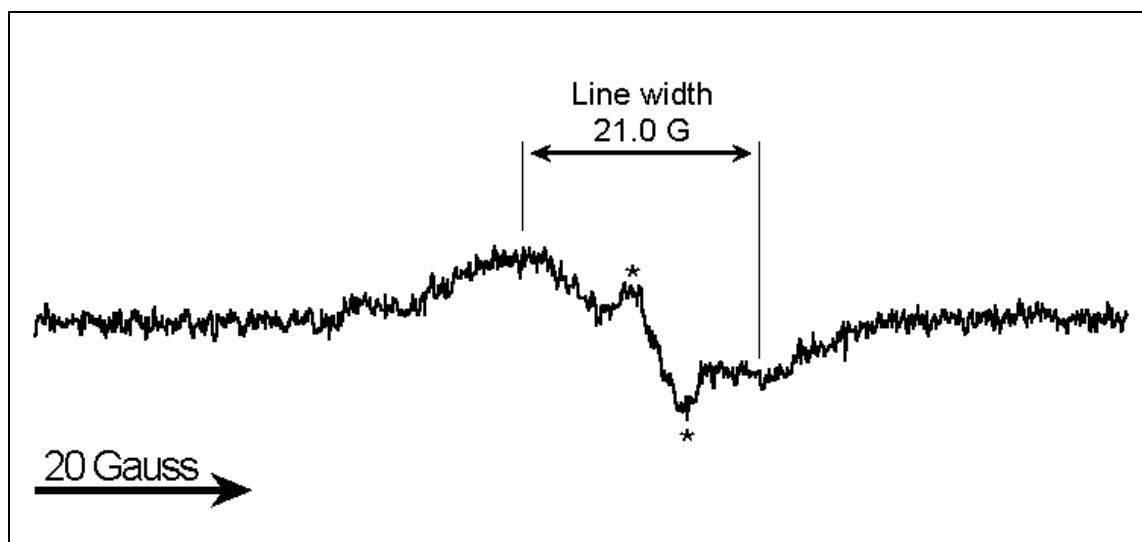


Figure 5.3

SSEPR spectrum of a solution containing 0.1 mM 2,2,6,6-tetramethylpiperidine-*N*-oxyl (TEMPO), 0.7 mM riboflavin, and 7 mM EDTA after full quenching of the signal from TEMPO.

It is evident from these results that irradiation of RF in the presence of an efficient one-electron donor leads to an appreciable concentration of the neutral semiquinone radical of RF, together with the corresponding cation radical derived from the one-electron donor. Conversely, if only RF is present, bimolecular triplet-triplet annihilation leads to a radical anion and a radical cation of RF via efficient one-electron transfer. Back-electron transfer, however, is almost equally efficient thereby leading to low radical concentrations - unnoticed in our investigations - both directly or via quenching of the TEMPO signal.

5.3 TIME-RESOLVED ELECTRON PARAMAGNETIC RESONANCE (TREPR)

For the time-resolved electron paramagnetic resonance (TREPR) investigations on sensitized irradiation of isohumulones and reduced derivatives, a similar set-up was used as described previously (**Chapter 2**). Some modifications were made in order to increase the sensitivity and time resolution (see 5.4). Furthermore, a pulsed Nd:YAG laser (third harmonic at 355 nm) was substituted for the excimer laser with a characteristic emission at 308 nm. On the other hand, the concentration of paramagnetic species was still too low to deliver a

significant TREPR signal. Therefore, flavin mononucleotide (FMN) was substituted for RF as a sensitizer, because of the significantly increased solubility due to incorporation of a phosphate group at the 5'-position. While carrying an identical chromophore, the photochemical characteristics of both compounds are very similar.

5.3.1 Investigations in model systems

As successfully applied in SSEPR experiments, TEMPO could also be used as a handle for indirect observation of paramagnetic species via the radical-triplet pair mechanism of CIDEP (**Chapter 2**). A mixture containing 10 mM flavin mononucleotide (FMN) and 1 mM TEMPO resulted in an expected intense three-line spectrum with a maximal intensity at a delay time of ca 750 ns, originating from polarization of the triplet energy levels of TEMPO in the collision with triplet-excited FMN (**Figure 5.4**).

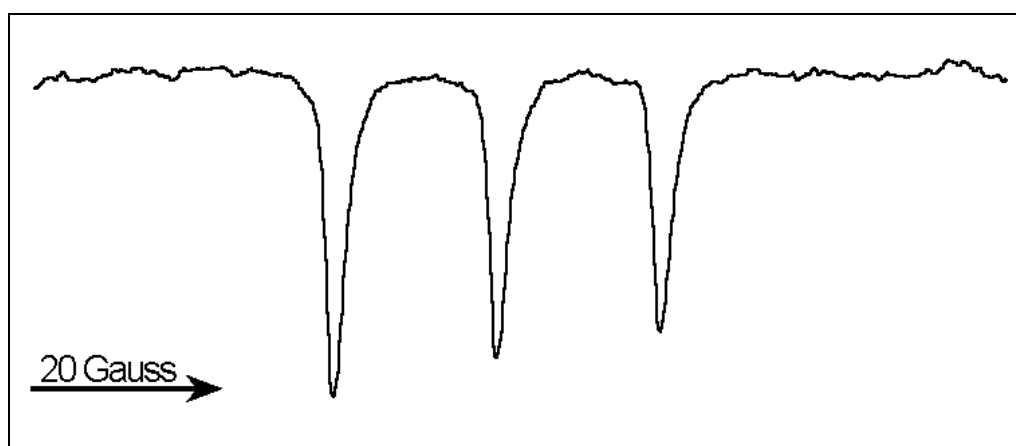


Figure 5.4

TREPR spectrum of a mixture containing 10 mM flavin mononucleotide (FMN) and 1 mM 2,2,6,6-tetramethylpiperidine-*N*-oxyl (TEMPO) recorded at a delay time of 750 ns

On irradiation of a 10 mM FMN-solution, a very weak, predominantly emissive, EPR signal was observed, while the intensity of the signal appeared enhanced at longer delay times (3-4 μ s). On the other hand, irradiation of a mixture containing 5 mM FMN and 50 mM EDTA resulted in a significant emissive/absorptive signal with a predominant emissive component, while a maximal amplitude was observed after a delay time of ca. 400 ns (**Figure 5.5**). The overall shape of the signal did not change over a time interval of 100 ns to 3 μ s. The linewidth of 19.0 G is consistent with formation of the neutral semiquinone radical. The

emissive/absorptive pattern stems from the radical-pair mechanism of CIDEP with triplet precursors.

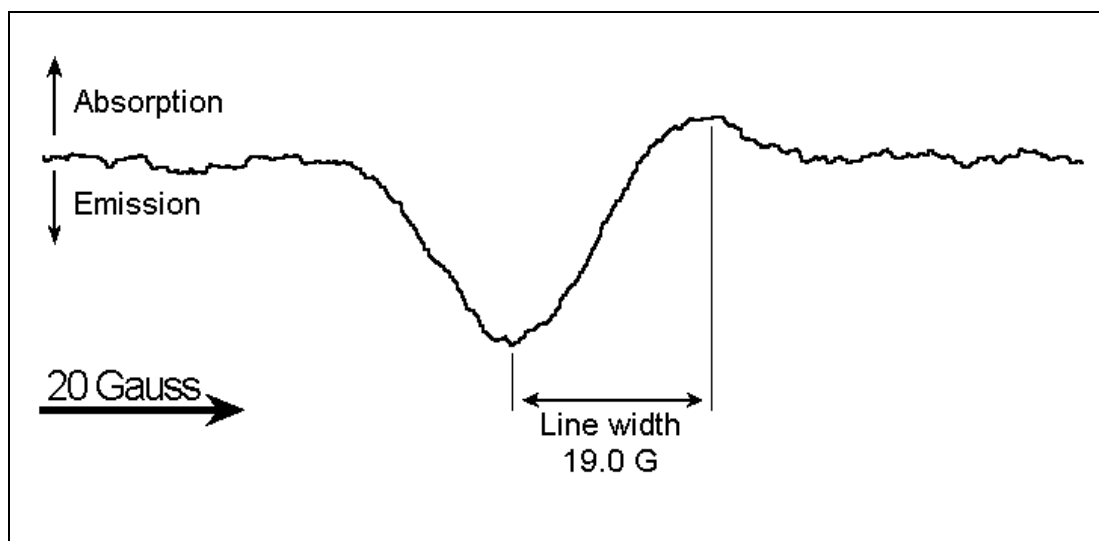


Figure 5.5

TREPR spectrum of a mixture containing 5 mM flavin mononucleotide (FMN) and 50 mM EDTA recorded at a delay time of 400 ns.

In agreement with the results described previously (**Chapter 4**), addition of an efficient one-electron donor such as EDTA rapidly leads to a significant concentration of radicals derived from one-electron transfer from the electron donor to triplet-excited FMN. In the absence of the efficient one-electron donor, triplet-triplet annihilation of flavins may result in one-electron transfer leading to an anion radical and a cation radical of the flavin. The concentration of radicals is, however, much lower due to efficient back-electron transfer and to the lower concentration of possible substrates for efficient one-electron transfer.

5.3.2 Investigations on isohumulones and reduced derivatives

Solutions containing 5 mM FMN and 10 mole equivalents of isohumulones (**5.2a-c**) (525 mg), tetrahydroisohumulones (**5.3a-c**) (539 mg), or dihydroisohumulones (**5.4a-c**) (530 mg), respectively, were prepared in a total volume of 30 mL (acetonitrile/water, 1/1, v/v). Another 8 mL of acetonitrile was added to the abovementioned samples to improve the solubility. TREPR spectra were recorded for all three samples over a time interval of 100 ns - 4 μ s.

Pulsed irradiation of the samples resulted in significant emissive/absorptive TREPR signals with a predominant emissive component. These signals appeared very similar to those observed for the model system comprising 5 mM FMN and 50 mM EDTA, while a maximal

amplitude was observed after a delay time of ca. 300 - 400 ns (**Figure 5.5**). On the other hand, a superimposed emissive signal was observed for isohumulones and tetrahydroisohumulones, while, for dihydroisohumulones, the broad emissive/absorptive EPR signal was not accompanied by a superimposed signal.

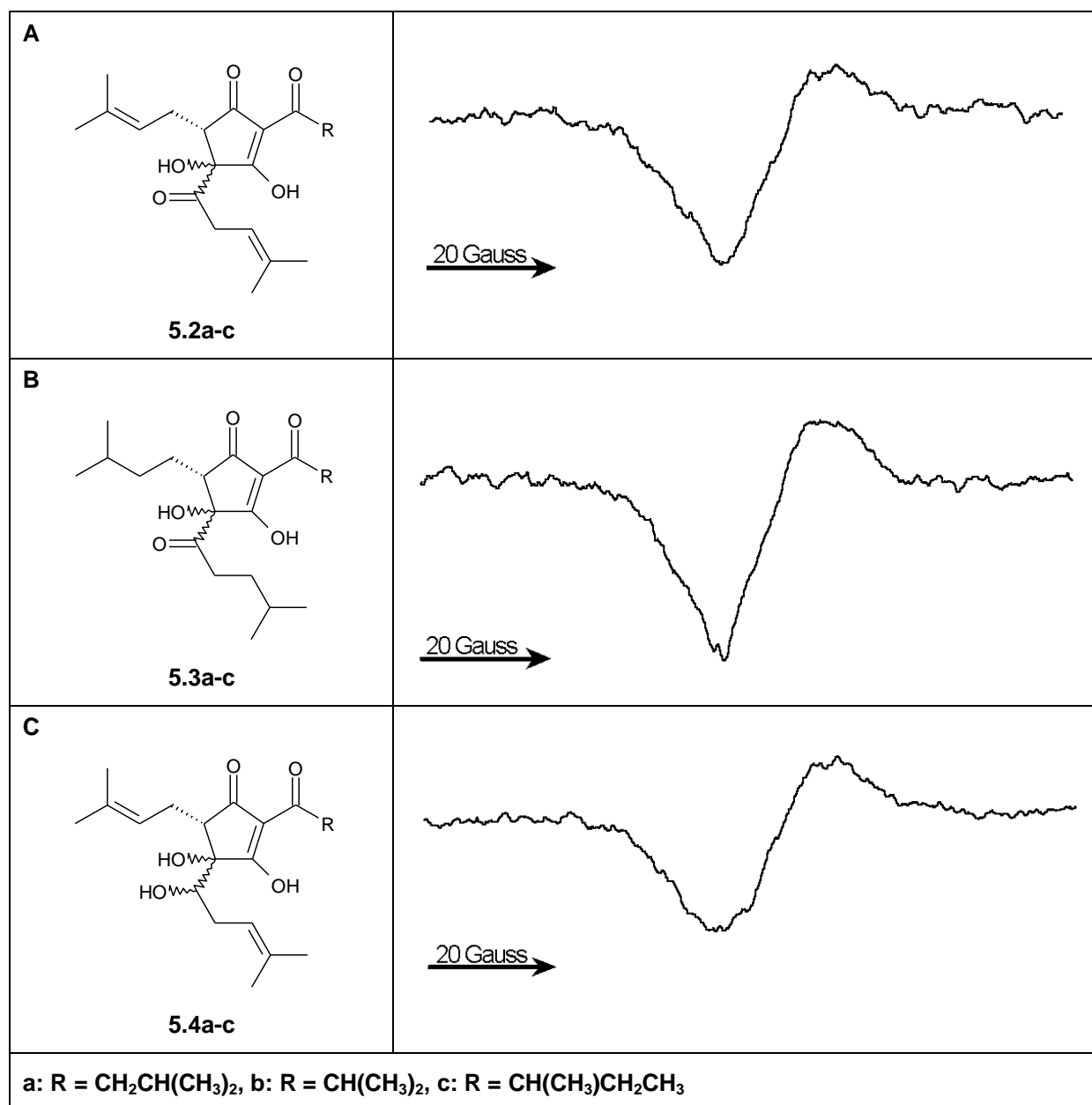


Figure 5.6

TREPR spectra of mixtures containing 5 mM flavin mononucleotide (FMN) and 50 mM of isohumulones (**A**), tetrahydroisohumulones (**B**), or dihydroisohumulones (**C**) on irradiation at 355 nm at a delay time of 300 ns.

The superimposed signals can be attributed to formation of secondary radicals, originating from degradation of the cation radicals formed via one-electron transfer from hop-derived

acids to triplet-excited FMN. When comparing the above results with the findings obtained on direct irradiation of isohumulones, tetrahydroisohumulones, and dihydroisohumulones (see Chapter 2), similarities are striking. The superimposed signal found on sensitized irradiation of isohumulones is significantly broader with respect to the superimposed signal observed on sensitized irradiation of tetrahydroisohumulones, with this difference clearly originating from the presence of either an insaturation or a saturation in the side chains. The narrowing of the signal is expected for secondary radicals with smaller and less significant hyperfine interactions. A 3-methylbut-2-enyl radical derived from degradation of the cation radical of isohumulones would lead to a much broader TREPR signal with respect to a 4-methylpentanoyl radical or a 3-methylbutyl radical derived from degradation of the cation radical of tetrahydroisohumulones. Evidence for the existence of these radicals follows from detailed product analysis on sensitized irradiation of isohumulones and their reduced derivatives (See Chapter 6). The absence of a superimposed signal during sensitized irradiation of dihydroisohumulones implies that no significant photodegradation of the corresponding cation radical occurs. Also, on direct irradiation, dihydroisohumulones did not give an EPR signal in TREPR experiments (See Chapter 2).

5.3.3 Investigations in the presence of cysteine

In addition to the samples with FMN and isohumulones, tetrahydroisohumulones, or dihydroisohumulones, respectively, a similar batch of samples was prepared, to which 25 mM cysteine was added in order to investigate the role of a sulfur source during formation of radicals on sensitized irradiation. On irradiation of a sample containing 5 mM FMN and 50 mM cysteine, again, an emissive/absorptive TREPR signal was observed (**Figure 5.6**), which was very similar to that recorded for a sample containing 5 mM FMN and 50 mM EDTA. Consequently, these TREPR spectra must also originate from one-electron transfer from cysteine to triplet-excited FMN. On the other hand, differences in signal amplitude and intensity versus delay time (maximal amplitude at ca. 800 ns rather than 400 ns) were noted. Since cysteine is a less efficient one-electron donor compared to EDTA, these variations seem reasonable.

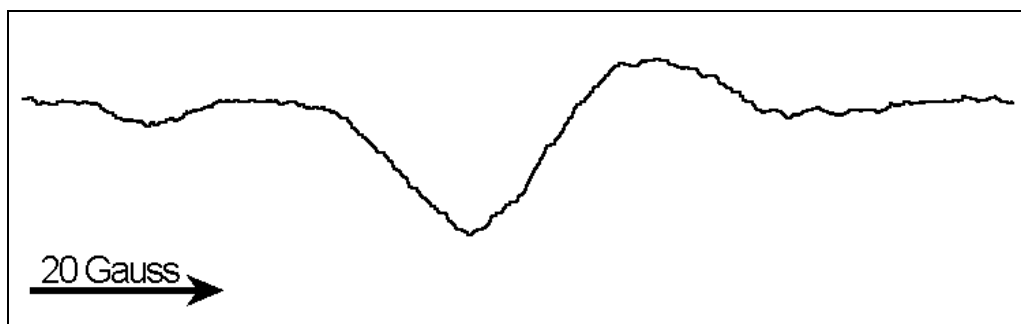


Figure 5.7

TREPR spectrum of a mixture containing 5 mM flavin mononucleotide (FMN) and 50 mM cysteine under pulsed irradiation at 355 nm recorded at a delay time of 800 ns.

Irradiation of samples containing 5 mM FMN, 25 mM cysteine, and 50 mM of the respective hop-derived acids resulted in TREPR spectra, which were very similar to those, found for the samples without addition of cysteine. These observations indicate that the sulfur source does not interfere significantly with primary photochemical events.

5.4 EXPERIMENTAL PART

Steady-State Electron Paramagnetic Resonance

Steady-state EPR experiments were carried out on a JEOL, USA, Inc. RE-1X EPR instrument operating at X-band frequency (9.5 GHz) interfaced with a personal computer for data acquisition. A cylindrical sample cell (id: 1 mm) was used to accommodate the polar solvents.

Time-Resolved Electron Paramagnetic Resonance

The experimental set-up for time-resolved electron paramagnetic resonance was already described in detail in Chapter 2. Some modifications were made for the experiments under sensitized irradiation conditions. A microwave amplifier (GaAs FET (noise figure 0.9 dB)) was positioned in front of the detector to increase the sensitivity and a wideband preamplifier (0.5-50 MHz) was arranged at the back of the detector to increase time resolution. Furthermore, a Double Balanced Mixer (DBM) was substituted for the Magic Tee with Crystal Microwave detector. The YAG-laser (Continuum Laser Powerlite Series) could deliver ca. 30 mJ/pulse at 355 nm.

5.5 REFERENCES

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- (5) Rustandi, R.R., Jorns, M.S.; *Photoinduced Spin-Polarized Radical Pair Formation in a DNA Photolyase Substrate Complex at Low Temperature*. Biochemistry, **1995**, *34*, 2284-2288.

CHAPTER 6

PHOTOREACTIVITY OF ISOHUMULONES AND REDUCED DERIVATIVES UNDER SENSITIZED IRRADIATION CONDITIONS

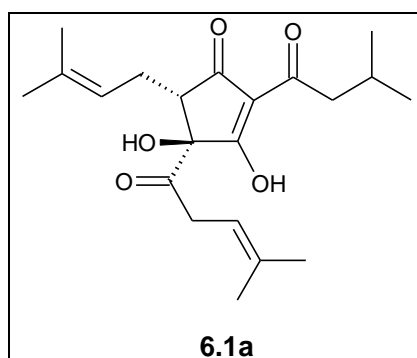
6.1 INTRODUCTION

Detailed product analyses of mixtures of photoreaction products derived from irradiation (at 300 nm) of isohumulones, tetrahydroisohumulones, and dihydroisohumulones, respectively, resulted in profound understanding of the primary photochemistry of these hop-derived acids (see Chapter 3). However, lightstruck flavor formation has been studied almost exclusively in model systems and real beer samples on exposure to visible light, with 350-500 nm being the most effective wavelength range. While isohumulones and their reduced derivatives are transparent at these wavelengths, the intermediacy of a sensitizer is required. Studies using model systems have shown unequivocally that riboflavin (RF; Vitamin B2) is able to sensitize the formation of lightstruck flavor in the presence of isohumulones and a suitable sulfur source. Moreover, several hundreds of ppm's of RF are present in beer,¹ hence it can safely be accepted that RF is a key element - although possibly not the unique factor - in the skunking of beer.

All previous investigations related to the formation of lightstruck flavor have invested on detection and quantification of 3-methylbut-2-ene-1-thiol (MBT),²⁻⁵ the major contributor to the off-flavor, while the fate of RF (as a sensitizer) or of isohumulones (as photosubstrates) on exposure to visible light has escaped attention. Comprehensive product analyses of mixtures of photoreaction products, derived from sensitized irradiation of solutions containing RF and isohumulones, tetrahydroisohumulones, or dihydroisohumulones, respectively, should give valuable information on the corresponding photoreactivities (in accordance with procedures described in Chapter 3). Combining the results from such photostationary experiments with those from photodynamic endeavors should lead to elaboration of relevant reaction schemes.

6.2 PHOTOREACTIVITY OF ISOHUMULONES UNDER SENSITIZED IRRADIATION CONDITIONS

In order to minimize the complexity of the mixtures of photoreaction products, individual isohumulones were preferred as photosubstrates. Addition of a small amount of RF (0.05 – 0.1 mole equivalents) was sufficient to induce complete photodegradation of isohumulones on prolonged irradiation (> 1 h). Irradiation of a solution (10 mL, methanol/water, 1/1) containing *trans*-isohumulone (**6.1a**, Scheme 6.1) (1.4 mM) and RF (0.066 mM) furnished a rather restricted number of major photoreaction products (**Figure 6.1**).



Scheme 6.1
Structure of *trans*-isohumulone (**6.1a**).

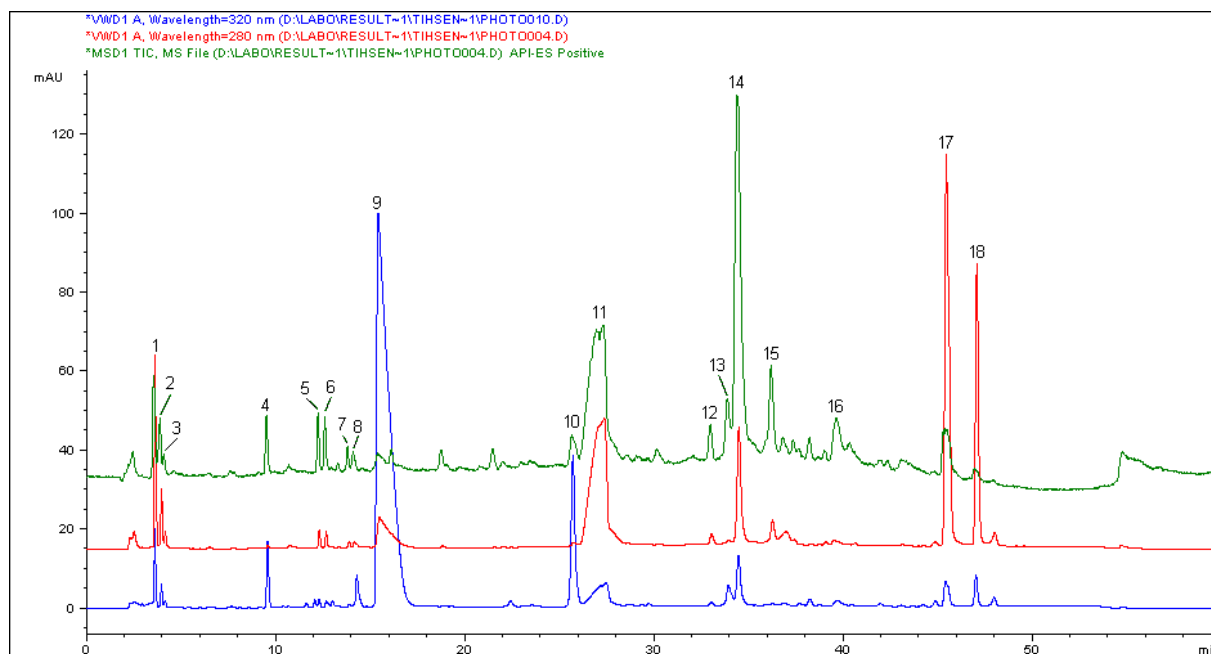


Figure 6.1

HPLC-MS analysis of a mixture of photoreaction products obtained on sensitized irradiation (visible light) of *trans*-isohumulone (**6.1a**) in the presence of riboflavin (**6.2**) for 1 h (—: UV-signal at 320 nm; —: UV-signal at 280 nm; —: positive ionization mode MS-signal).

An overview of characterized constituents and their corresponding molecular weights is given in **Table 6.1**. Photoreaction products eluting in the most polar part of the chromatogram with retention times between 3 min and 15 min are all riboflavin (RF) derivatives, while compounds eluting with retention times between 15 min and 50 min originate from sensitized photodegradation of *trans*-isohumulone.

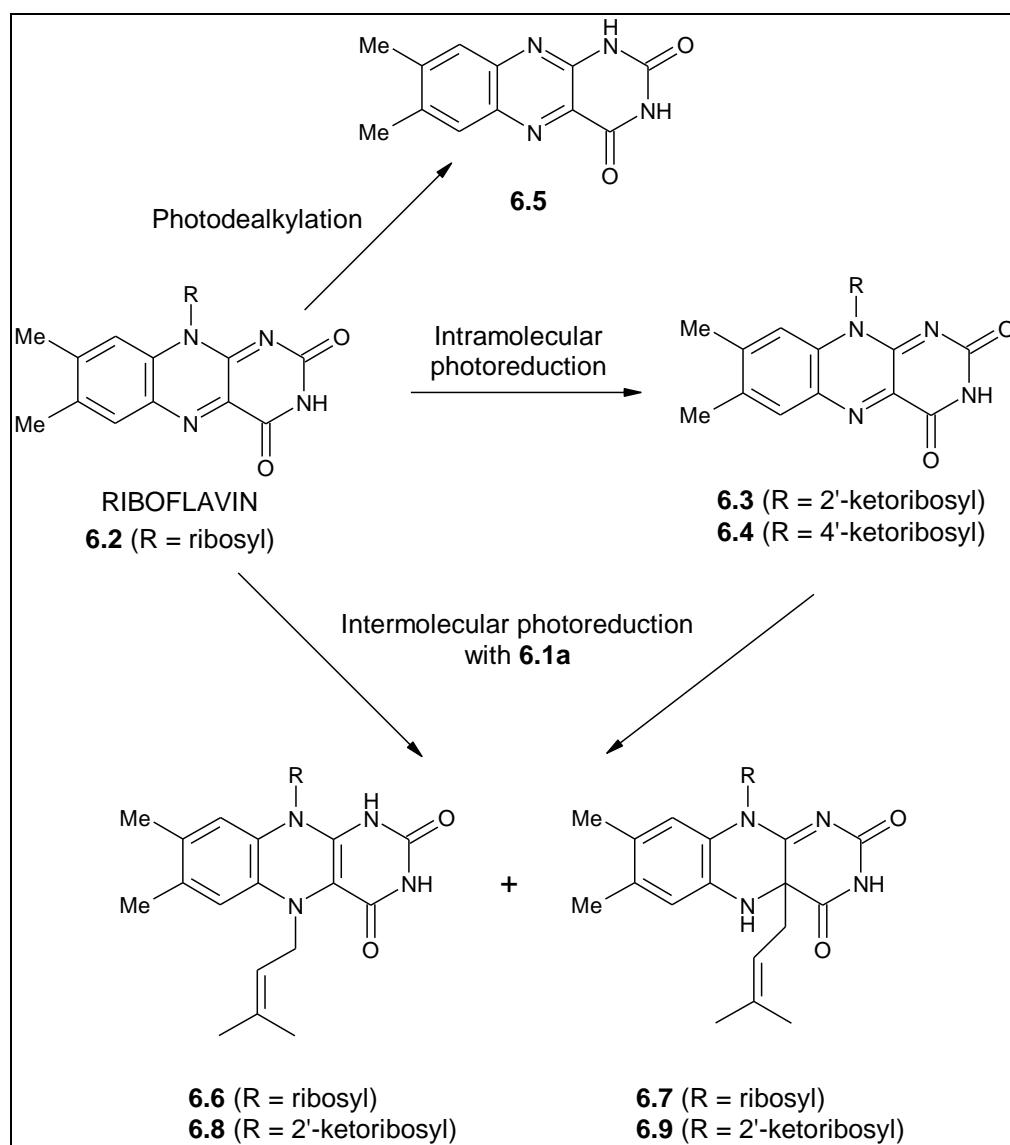
Table 6.1

Overview of characterized compounds on HPLC-MS-analysis of the mixture of photoreaction products formed on sensitized irradiation (visible light) of *trans*-isohumulone (**6.1a**) in the presence of riboflavin (**6.2**) (*a: with respect to **6.2**; b: with respect to **6.1a**).

| Peak nr. | Retention time/min | Molecular weight/amu | Δ MW/amu* | Compound |
|----------|--------------------|----------------------|-------------------|--------------|
| 1 | 3.62 | 376 | 0 ^a | 6.2 |
| 2 | 3.95 | 374 | -2 ^a | 6.3 |
| 3 | 4.16 | 374 | -2 ^a | 6.4 |
| 4 | 9.57 | 242 | -134 ^a | 6.5 |
| 5 | 12.30 | 446 | 70 ^a | 6.6 |
| 6 | 12.66 | 446 | 70 ^a | 6.7 |
| 7 | 13.88 | 444 | 68 ^a | 6.8 |
| 8 | 14.15 | 444 | 68 ^a | 6.9 |
| 9 | 15.49 | 280 | -82 ^b | 6.10a |
| 10 | 25.71 | 296 | -66 ^b | - |
| 11 | 27.17 | 362 | 0 ^b | 6.1a |
| 12 | 33.05 | 362 | 0 ^b | - |
| 13 | 33.96 | 316 | -46 ^b | - |
| 14 | 34.44 | 334 | -28 ^b | 6.11a |
| 15 | 36.25 | 394 | 32 ^b | - |
| 16 | 39.71 | 316 | -46 ^b | - |
| 17 | 45.44 | 362 | 0 ^b | 6.12a |
| 18 | 47.06 | 362 | 0 ^b | 6.12a |

6.2.1 Photoreaction products derived from riboflavin

Irradiation of RF (**6.2**) (**Scheme 6.2**) ($t_R = 3.62$ min, MW = 376 amu) resulted in photoreaction products such as 2'-ketoflavin (**6.3**) ($t_R = 3.95$ min, MW = 374 amu) and 4'-ketoflavin (**6.4**) ($t_R = 4.16$ min, MW = 374 amu) via intramolecular photoreduction proceeding by abstraction of the α -hydrogen at C-2' or C-4', respectively, followed by reoxidation of the isoalloxazine moiety.⁵ Photodealkylation of RF led to lumichrome (**6.5**) (MW = 242 amu)⁶ with concurrent characteristic alteration of the UV features from isoalloxazine ($\lambda_{max} = 450$ nm) to alloxazine ($\lambda_{max} = 350$ nm), found for a compound eluting with $t_R = 9.57$ min.

**Scheme 6.2**

Formation of photoreaction products derived from riboflavin (**6.2**) on sensitized irradiation (visible light) of *trans*-isohumulone (**6.1a**) in the presence of riboflavin.

Furthermore, four compounds eluting as pairs of peaks at ca. 12.5 min with a molecular weight of 446 amu and at ca. 14 min with a molecular weight of 444 amu, respectively, were characterized as flavin derivatives. The increase in molecular weight by 70 amu relative to RF and 2'-ketoflavin, respectively, implies addition of a 3-methylbut-2-enyl group and can be envisaged to proceed via intermolecular photoreduction.⁶ In a first step, one-electron transfer from the ground state of *trans*-isohumulone (**6.1a**) to triplet-excited RF yields a semiquinone radical in combination with a cation radical of *trans*-isohumulone (or a neutral radical after deprotonation). Subsequent degradation of the cation radical of *trans*-isohumulone affords a 3-methylbut-2-enyl radical (either directly or after rapid decarbonylation of a 4-methylpent-3-

enoyl radical) via formal α -cleavage of the side chain at C-4 (see 6.2.2). Finally, recombination of the 3-methylbut-2-enyl radical with a semiquinone radical either from riboflavin or 2'-ketoflavin gives the observed addition products **6.6/6.7** and **6.8/6.9**, respectively. The pairs of isomeric compounds observed in our experiments are most likely due to regiodifferentiated addition of a 3-methylbut-2-enyl radical at *N*-5 or C-4a. It has been reported that, depending on substrate, temperature, solvent, and pH, C-4a-, *N*-5-, or C-8 adducts may be formed on intermolecular photoreduction of flavins.^{6,7}

6.2.2 Photoreaction products derived from *trans*-isohumulone

A bathochromic shift of ca. 50 nm with respect to the UV spectrum of *trans*-isohumulone ($\lambda_{\text{max}} = 280$ nm) was observed for a major photoreaction product eluting with $t_R = 15.49$ min ($\lambda_{\text{max}} = 330$ nm), suggesting extended conjugation with respect to *trans*-isohumulone (**6.1a**). The molecular weight of 280 amu indicates formal loss of the 4-methylpent-3-enoyl side chain at C-4 resulting in cyclopent-2-ene-1,4-dione derivative **6.10a**. This photoreaction product was also formed under direct irradiation conditions, albeit in very low yield (see 3.3.5).

Moreover, a major photoreaction product eluting with $t_R = 34.44$ min was found to be identical with a minor photoreaction product derived from direct irradiation of *trans*-isohumulone (**6.1**) (see 3.3.1). The molecular weight of 334 amu is consistent with compound **6.11a** (Scheme 6.3), formed by formal loss of CO ($\Delta = 28$ amu with respect to **6.1a**). On ionization in the negative MS-mode, two complementary daughter ions were observed at 125 amu $[\text{C}_7\text{H}_9\text{O}_2]^-$ and 207 amu $[\text{C}_{13}\text{H}_{19}\text{O}_2]^-$, respectively. These ions are indicative of fragmentation of the molecular ion $[\text{M-H}]^-$ (formed on deprotonation of **6.11a**) to a fragment ion containing the saturated side chain $[\text{D}_1\text{-H}]^-$ and a fragment ion containing both unsaturated side chains $[\text{D}_2\text{-H}]^-$ (Figure 6.2).

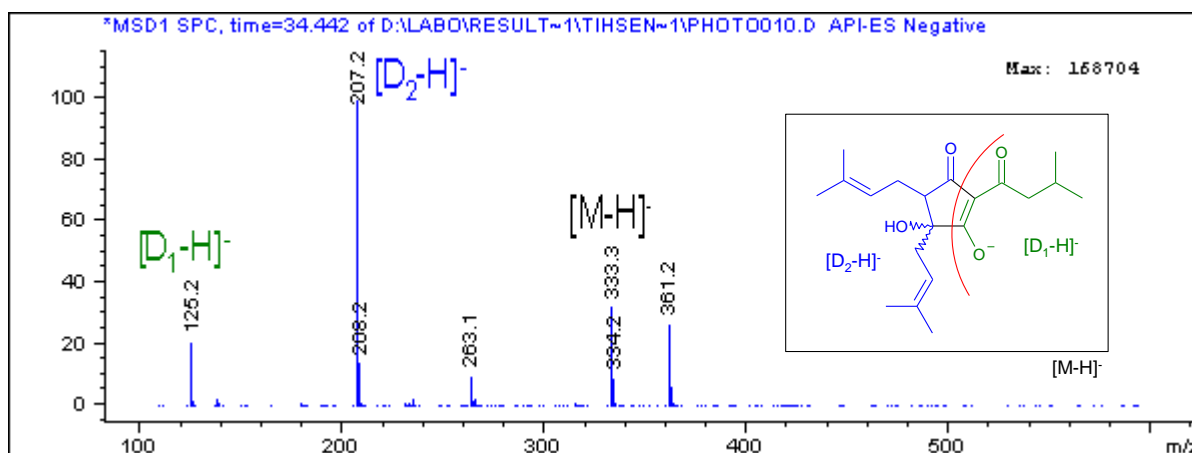
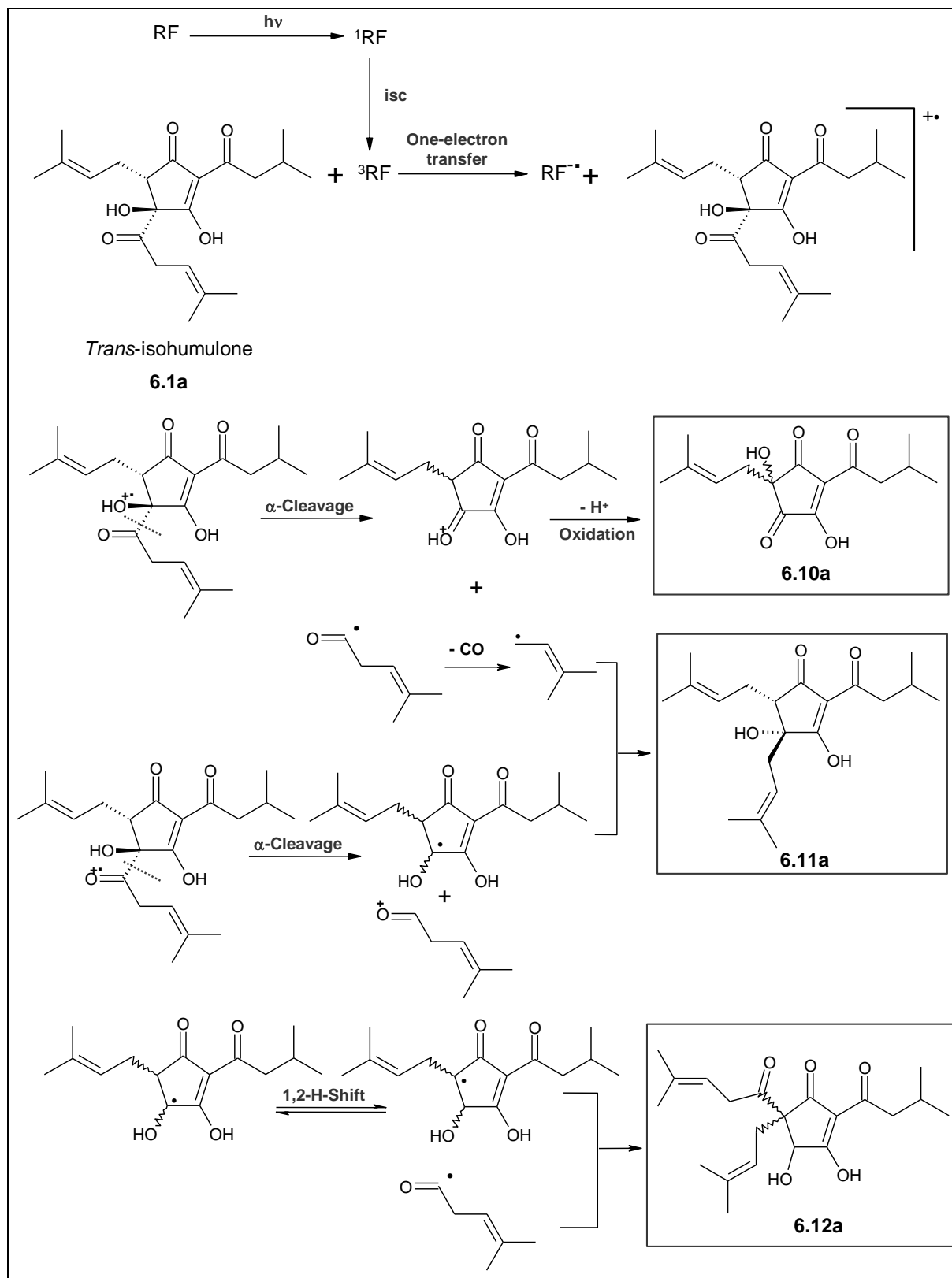


Figure 6.2

Mass spectrum of compound **6.11a** (Scheme 6.3) eluting with $t_R = 34.44$ min on HPLC-MS analysis (peak 14 in Figure 6.1).

Apparently, one-electron transfer from *trans*-isohumulone (**6.1a**) to triplet-excited RF results in a semiquinone radical along with an unstable cation radical (or neutral radical after deprotonation) derived from *trans*-isohumulone. It remains to be determined from which functional group of *trans*-isohumulone (**6.1a**) the electron is transferred. In principle, removal of a non-bonded electron should be considered. The electron-rich β -tricarbonyl chromophore could deliver an electron, but loss of a proton and recapture of a hydrogen atom by the resulting stabilized triacylmethyl radical is expected to reinstall the β -tricarbonyl moiety. This reasoning is supported by the UV-spectra of all major photoreaction products indicating that the β -tricarbonyl chromophore has been retained. On the other hand, electron abstraction from the tertiary alcohol group and α -cleavage leads to a 4-methylpent-3-enoyl radical and a protonated five-membered ring structure. Deprotonation should furnish dehydrohumulinic acid (see 1.1.3), but, instead, oxidized dehydrohumulinic acid **6.10a** (Scheme 6.3) was identified. Formation of this compound is very likely the result of the abstraction of the activated methine hydrogen in dehydrohumulinic acid, followed by oxidation. Alternatively, an electron could be released from the carbonyl group in the side chain at C-4 and subsequent α -cleavage would furnish, in addition to an acylium ion, a ketyl radical. Recombination with a 3-methylbut-2-enyl radical gives rise to formation of decarbonylated isohumulone (**6.11a**, Scheme 6.3). Since only one compound with a molecular weight of 334 amu was present in the mixture of photoreaction products (see Table 6.1), it is accepted that the more stable *cis*-epimer is formed. The prevalence of **6.11a** clearly detracts from formation of dimeric-type compounds, which were present indeed in mixtures of photoreaction products obtained on direct irradiation of *trans*-isohumulone (**6.1a**) (see 3.3.2)



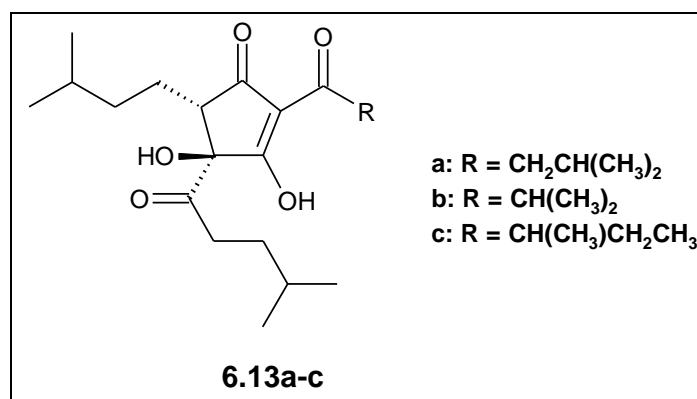
Scheme 6.3

Formation of major photoreaction products derived from sensitized irradiation (visible light) of *trans*-isohumulone (6.1a) in the presence of riboflavin.

Two compounds, eluting in the least polar part of the chromatogram with retention times of 45.44 min and 47.06 min, respectively, were isomeric with *trans*-isohumulone (6.1a). Since humulone could be excluded on the basis of the retention time and UV features (both compounds have a UV spectrum similar to that of *trans*-isohumulone), establishment of the nature of these isomers needs further investigation. An intriguing possibility could be formation of diastereomers such as 6.12a (Scheme 6.3) involving 1,2-hydrogen shift within the abovementioned five-membered ketyl radical and recombination with a 4-methylpent-3-enoyl radical. Characterization of minor photoreaction products was not attempted.

6.3 PHOTOREACTIVITY OF TETRAHYDROISOHUMULONES UNDER SENSITIZED IRRADIATION CONDITIONS

The chromatogram of the mixture of photoreaction products resulting from sensitized irradiation of *trans*-tetrahydroisohumulones (**6.13a-c**, **Scheme 6.4**) (2.7 mM) in the presence of ca. 0.05 mole equivalents of riboflavin (0.13 mM) shows evident similarities to the chromatogram, obtained on analysis of the mixture of photoreaction products formed on sensitized irradiation of *trans*-isohumulone (**Figure 6.3**). Scrutinous analysis revealed minor, yet interesting differences. An overview of the characterized constituents and their corresponding molecular weights is given in **Table 6.2**. In analogy to sensitized irradiation of *trans*-isohumulone, photoreaction products can be divided in two series. All riboflavin-derived photoreaction products elute in the most polar part of the chromatogram with retention times between 3 min and 16 min, while photoreaction products originating from *trans*-tetrahydroisohumulones elute with retention times between 15 min and 52 min.



Scheme 6.4
Structures of *trans*-tetrahydroisohumulones (**6.13a-c**).

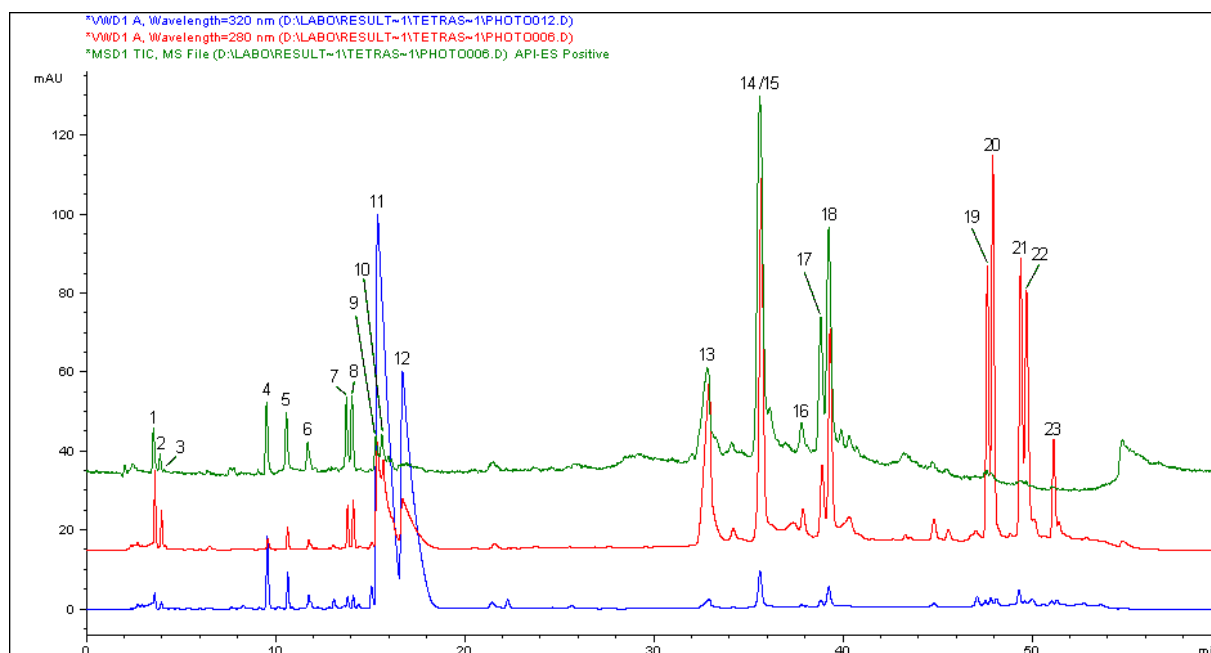


Figure 6.3

HPLC-MS analysis of a mixture of photoreaction products obtained on sensitized irradiation (visible light) of *trans*-tetrahydroisohumulones (**6.13a-c**) in the presence of riboflavin for 1h (—: UV-signal at 320 nm; —: UV-signal at 280 nm; —: positive ionization mode MS-signal).

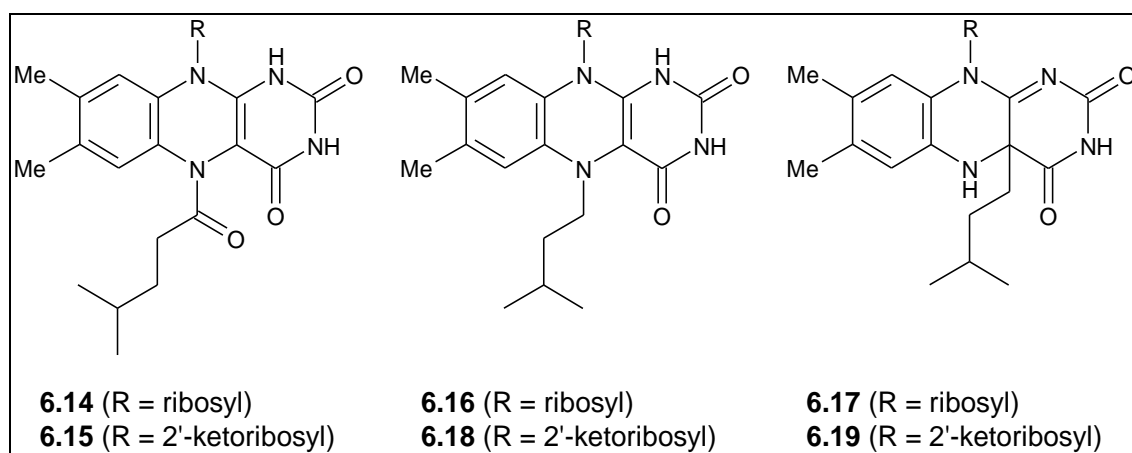
Table 6.2

Overview of characterized compounds on HPLC-MS-analysis of the mixture of photoreaction products formed on sensitized irradiation (visible light) of *trans*-tetrahydroisohumulones (**6.13a-c**) in the presence of riboflavin (**6.2**) (*a: with respect to **6.2**; b: with respect to the appropriate constituent of **6.13a-c**).

| Peak nr. | Retention time/min | Molecular weight/amu | Δ MW/amu* | Compound |
|----------|--------------------|----------------------|-------------------|--------------|
| 1 | 3.61 | 376 | 0 ^a | 6.2 |
| 2 | 3.95 | 374 | -2 ^a | 6.3 |
| 3 | 4.14 | 374 | -2 ^a | 6.4 |
| 4 | 9.58 | 242 | -134 ^a | 6.5 |
| 5 | 10.63 | 476 | 100 ^a | 6.14 |
| 6 | 11.75 | 474 | 98 ^a | 6.15 |
| 7 | 13.80 | 448 | 72 ^a | 6.16 |
| 8 | 14.10 | 448 | 72 ^a | 6.17 |
| 9 | 15.37 | 446 | 70 ^a | 6.18 |
| 10 | 15.38 | 268 | -84 ^b | 6.20b |
| 11 | 15.69 | 446 | 70 ^a | 6.19 |
| 12 | 16.69 | 282 | -84 ^b | 6.20a |
| 13 | 32.87 | 352 | 0 ^b | 6.13b |
| 14 | 35.59 | 324 | -28 ^b | 6.21b |
| 15 | 35.59 | 366 | 0 ^b | 6.13a |
| 16 | 37.77 | 366 | 0 ^b | 6.13c |
| 17 | 38.82 | 366 | 0 ^b | - |
| 18 | 39.23 | 338 | -28 ^b | 6.21a |
| 19 | 47.61 | 352 | 0 ^b | 6.22b |
| 20 | 47.90 | 352 | 0 ^b | 6.22b |
| 21 | 49.37 | 366 | 0 ^b | 6.22a |
| 22 | 49.68 | 366 | 0 ^b | 6.22a |
| 23 | 51.12 | 366 | 0 ^b | 6.22c |

6.3.1 Photoreaction products derived from riboflavin

The four first eluting compounds in the mixture of photoreaction products obtained on sensitized irradiation of *trans*-tetrahydroisohumulones (**6.13a-c**) are identical to those derived from sensitized irradiation of *trans*-isohumulone (**6.1a**). In addition to residual RF (**6.2**) ($t_R = 3.61$ min), 2'-ketoflavin (**6.3**) ($t_R = 3.95$ min), 4'-ketoflavin ($t_R = 4.14$ min) (**6.4**), and lumichrome (**6.5**) ($t_R = 9.58$ min), were observed as photoreaction products derived from direct photodegradation of RF. Also, a number of RF derivatives with an increased molecular weight resulting from intermolecular photoreduction could be detected. RF adducts formed on sensitized irradiation of *trans*-tetrahydroisohumulones were found to include recombination products of a semiquinone radical with a 4-methylpentanoyl radical, as well as with a 3-methylbutyl radical. The molecular weights of 476 amu and 474 amu found for two compounds eluting with $t_R = 10.63$ min and $t_R = 11.75$ min, respectively, are consistent with incorporation of a 4-methylpentanoyl group via intermolecular photoreduction in RF (**6.15**) and 2'-ketoflavin (**6.16**), respectively (**Scheme 6.5**). Most likely, regioselective addition of a 4-methylpentanoyl group to *N*-5 occurred, while no isomeric pairs were observed. On the other hand, the presence of isomeric photoreaction products with molecular weights of 448 amu and 446 amu eluting with retention times of ca. 13.80 min and ca. 15.69 min, respectively, indicates regiodifferentiated addition of a 3-methylbutyl group, most likely, at *N*-5 and C-4a in RF (**6.16-6.17**) and 2'-ketoflavin (**6.18-6.19**), respectively.^{6,7}



Scheme 6.5

Photoreaction products resulting from intermolecular photoreduction of riboflavin in the presence of *trans*-tetrahydroisohumulones (**6.13a-c**).

6.3.2 Photoreaction products derived from *trans*-tetrahydroisohumulones

Sensitized irradiation of *trans*-tetrahydroisohumulones (**6.13a-c**) in the presence of RF resulted in a rather restricted number of major photodegradation products corresponding to analogous major photoreaction products observed on sensitized irradiation of *trans*-isohumulone (**6.1a**) in the presence of RF. Two major photoreaction products eluting with retention times of 15.38 min and 16.69 min, respectively, were characterized as analogs of **6.10a**. A decrease in molecular weight with 84 amu with respect to **6.13a** and **6.13b** is consistent with formal loss of the 4-methylpentanoyl side chain at C-4 and concurrent oxidation leading to cyclopent-2-ene-1,4-dione derivatives **6.20a** and **6.20b**, respectively (**Scheme 6.6**). Molecular weights of 324 amu and 338 amu found for peaks eluting with retention times of 35.59 min and 39.23 min, respectively, are consistent with compounds such as **6.21a** and **6.21b** formed via formal loss of CO in analogy to **6.11a**. Again, on ionization in the negative MS-mode, fragmentation of the molecular ions $[M-H]^-$ resulted in two complementary daughter ions. For both **6.21a** and **6.21b**, an identical fragment ion containing the side chains at C-4 and C-5 was observed with a mass of 211 amu ($[D_2-H]^-$), while the masses for the fragment ions containing the side chain at C-2 ($[D_1-H]^-$) were found at 111 amu and 125 amu, respectively (**Figure 6.4**).

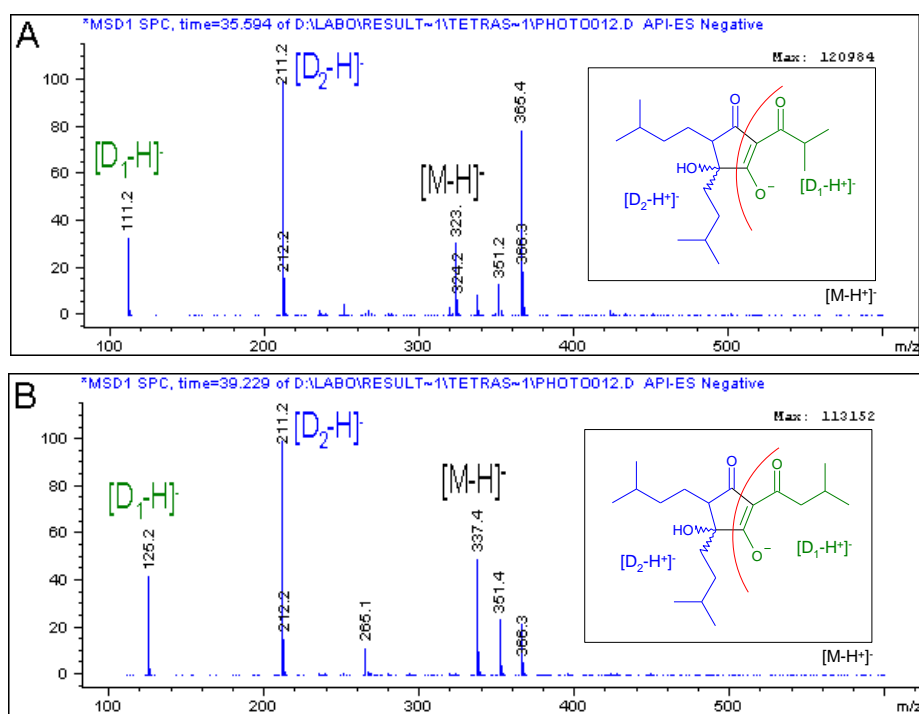
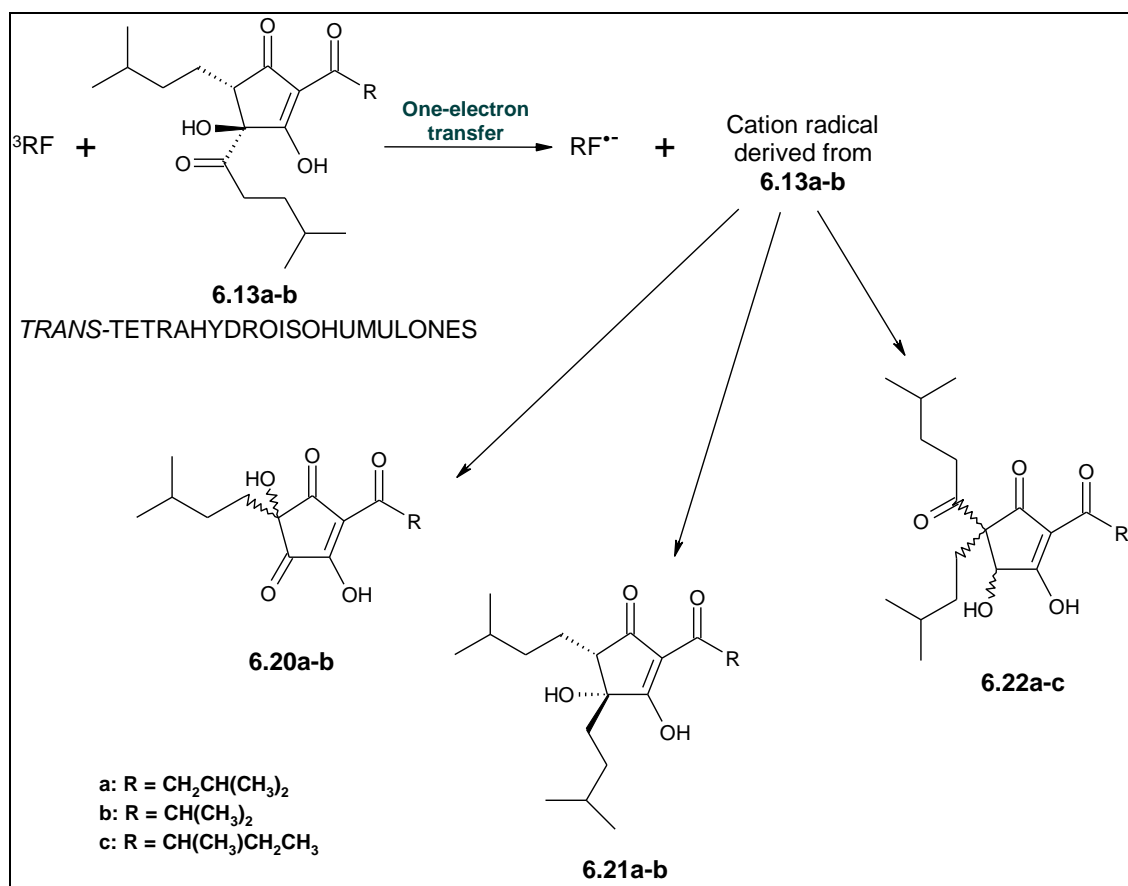


Figure 6.4

Mass spectra of decarbonylated tetrahydroisohumulones (**6.21a-b**) (**Scheme 6.6**) eluting with $t_R = 35.59$ min (**A**) and 39.22 min (**B**), respectively, on HPLC-MS-analysis (peaks 15 and 18, respectively, in **Figure 6.3**).

Furthermore, in the least polar part of the chromatogram compounds eluting as pairs with retention times between 47.61 min and 51.12 min were found to be isomeric with **6.13a** and **6.13b**, respectively. In view of the UV spectra closely resembling those of **6.13a-c**, one or the other diastereomer of **6.22a-c** could have been formed in analogy to the observed formation of **6.12a** on sensitized irradiation of **6.1a**. The discussion elaborated to account for formation of compounds **6.10a**, **6.11a**, and **6.12a** (see 6.2.2) can be repeated for formation of compounds **6.20a-b**, **6.21a-b**, and **6.22a-c**, respectively.



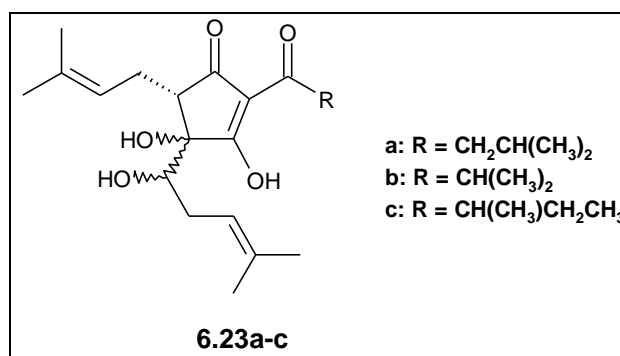
Scheme 6.6

Photoreaction products derived from *trans*-tetrahydroisohumulones (**6.13a-c**) on sensitized irradiation (visible light) in the presence of riboflavin.

6.4 PHOTOREACTIVITY OF DIHYDROISOHUMULONES UNDER SENSITIZED IRRADIATION CONDITIONS

In contrast to the rapid photolyses of both *trans*-isohumulone (**6.1a**) and *trans*-tetrahydroisohumulones (**6.13a-c**) in conjunction with formation of a restricted number of

major photoreaction products, dihydroisohumulones (**6.23a-c**, **Scheme 6.7**) were photostable under conditions of sensitized irradiation in the presence of RF (**Figure 6.5**). Although results from TREPR and laser-flash photolysis transient absorption spectroscopy clearly show the occurrence of one-electron transfer from dihydroisohumulones to triplet-excited RF, subsequent degradation of the incipient cation radical apparently does not occur. This observation is consistent with the results obtained by TREPR, while no superimposed signals were found on sensitized irradiation of dihydroisohumulones. Efficient back-electron transfer may result in reformation of the photosubstrates, hence photodegradation is avoided. It can be stated that the carbonyl group in the side-chain at C-4 is essential for efficient decomposition of the cation radical formed on one-electron transfer from isohumulones and tetrahydroisohumulones to triplet-excited RF.



Scheme 6.7
Structures of dihydroisohumulones (**6.23a-c**).

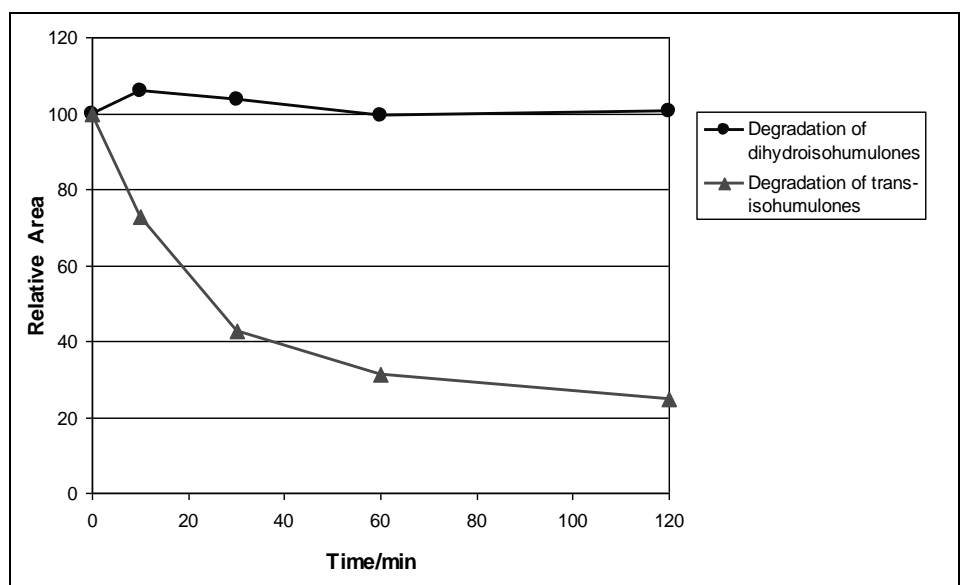


Figure 6.5
Photosensitized degradation of *trans*-isohumulone (**6.1a**) and dihydroisohumulones (**6.23a-c**) in the presence of riboflavin.

6.5 EXPERIMENTAL PART

Irradiation conditions

Trans-isohumulone (1.4 mM), a mixture of *trans*-tetrahydroisohumulones (2.7 mM), or dihydroisohumulones (2.7 mM) were irradiated in a deaerated mixture of methanol/water (1/1, v/v, 10 mL) containing 0.05 mole equivalents of riboflavin with visible light (photoreactor containing 4 Philips Cool White Lamps of 8 W) at varying exposure times (up to 8 h). Deaeration of the samples was performed by flushing with solvent saturated N₂ for ca. 20 min.

Remark: A detailed description of sample preparation, chromatographic conditions, and mass spectral settings can be found in the experimental part of Chapter 3 (see 3.6).

6.6 REFERENCES

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ANNEX

In a final chapter, a fully innovative method is presented to inhibit formation of the lightstruck flavor in beer based on the results of this Ph.D. project. The research contract d.d. 1st of March 1997 between Cobrew N.V., Leuven, and Ghent University stipulates (page 4, item 7 “Proprietary rights”; page 5, item 7a “Confidential information”) that any and all inventions with potential industrial applications must not be made available to the public before a patent is filed. As patent application is in preparation, no results can be disclosed at this time. However, if any member of the reading and examination commissions should wish to consult the chapter at his discretion, he may do so by personal inspection with the author and the promoter after signing a confidentiality agreement.

Ghent, 14 May 2001

Arne Heyerick

Prof. Dr. D. De Keukeleire

Summary

An objectionable off-flavor is produced on exposure of beer to light. Current understanding regarding formation of the lightstruck flavor (LSF) in beer dates from the original reports by Kuroiwa et al. in the early sixties. Using model systems, they showed that LSF was produced in a light-induced reaction involving riboflavin as a sensitizer (riboflavin is the main flavin in beer), isohumulones (hop-derived, beer-bittering compounds), and a sulfur source (e.g. cysteine or sulfur-rich proteins). The off-flavor was attributed to formation of 3-methylbut-2-ene-1-thiol (MBT). This mechanism has been frequently referred to in articles dealing with light-induced off-flavors in beer and all relevant data collected to date do not contradict the Kuroiwa-premise. Although the key components are known for more than three decades, details on the mechanism of interaction between these components are lacking.

This Ph.D. project aimed at obtaining a thorough and detailed understanding of the mechanism of formation of LSF in beer. The issue was approached by an in-depth investigation of the interacting components on a molecular level using model systems, consisting of isohumulones, dihydroisohumulones, or tetrahydroisohumulones in the presence or absence of riboflavin or flavin mononucleotide as a sensitizer.

Unraveling a photochemical mechanism implies studies in various time domains. Thus, model systems were subjected to both photostationary irradiation and photodynamic flash-initiated experiments. Information on the nature of intervening photo-excited states and intermediates, along with mechanistic features, were retrieved from time-resolved electron paramagnetic resonance (TREPR) and flash photolysis transient absorption spectroscopy (TAS). On characterization and identification of prevalent photoreaction products by comprehensive product analysis, relevant reaction schemes were elaborated to account for formation of key compounds.

In a first section, a detailed study of the photolability of isohumulones, dihydroisohumulones, and tetrahydroisohumulones, is described. On laser-flash photolysis at 308 nm, isohumulones and tetrahydroisohumulones yielded strong emissive TREPR signals (originating from the triplet mechanism of chemically induced dynamic electron polarization), while, under identical conditions, no TREPR signal was observed for dihydroisohumulones. This finding provides the first direct spectroscopic evidence for the resistance of dihydroisohumulones to photolysis and confirms conclusively that radicals produced on

photolysis of isohumulones and tetrahydroisohumulones originate from the α -hydroxyketone chromophore as present in isohumulones and tetrahydroisohumulones.

Using well-substantiated simulations, the most prevalent radicals on photolysis of isohumulones were characterized as a five-membered ring ketyl radical in conjunction with a 3-methylbut-2-enyl radical, while photolysis of tetrahydroisohumulones led to production of a similar five-membered ring ketyl radical and a 4-methylpentanoyl radical. Combined TREPR data allowed to propose a mechanism for photodegradation of isohumulones and tetrahydroisohumulones on direct irradiation. Absorption of UV light by the β -tricarbonyl chromophore leads to the excited singlet state. After intersystem crossing to the excited triplet state, intramolecular triplet energy transfer occurs to the isolated α -hydroxyketone, which in the excited state ultimately undergoes Norrish Type I α -cleavage, thereby furnishing the radicals as detailed above. Comprehensive product analyses of complex mixtures of photoreaction products derived from isohumulones and tetrahydroisohumulones showed that recombination of the most prevalent radicals as characterized by TREPR accounts for formation of the majority of photoreaction products, while minor photoreaction products were found to originate from other pathways. The main reaction products on direct irradiation of isohumulones were characterized as decarbonylated dehydrated isohumulones, while the predominant reaction products on direct irradiation of tetrahydroisohumulones were identified as dimeric-type compounds formed on recombination of two five-membered ring ketyl radicals.

In a second section, the interaction between isohumulones, dihydroisohumulones, and tetrahydroisohumulones with riboflavin or flavin mononucleotide on sensitized irradiation was investigated in detail. Laser-flash photolysis at 355 nm of mixtures containing either isohumulones, dihydroisohumulones or tetrahydroisohumulones, and riboflavin resulted in transient absorption spectra (350-800 nm) dominated by the absorption of a neutral flavin semiquinone radical, while the absorption of triplet-excited riboflavin decreased very rapidly. These observations are consistent with a one-electron transfer from the ground state of either isohumulones, dihydroisohumulones or tetrahydroisohumulones to the excited triplet state of riboflavin. The feasibility of one-electron transfer derives from consideration of the redox potentials of the interacting species. The redox potential of riboflavin shifts from -0.3 V vs. NHE in the ground state to +1.7 V vs. NHE in the excited triplet state. Furthermore, TREPR signals on laser-flash photolysis of mixtures containing isohumulones or tetrahydroisohumulones, in addition to flavin mononucleotide, showed a superimposed signal

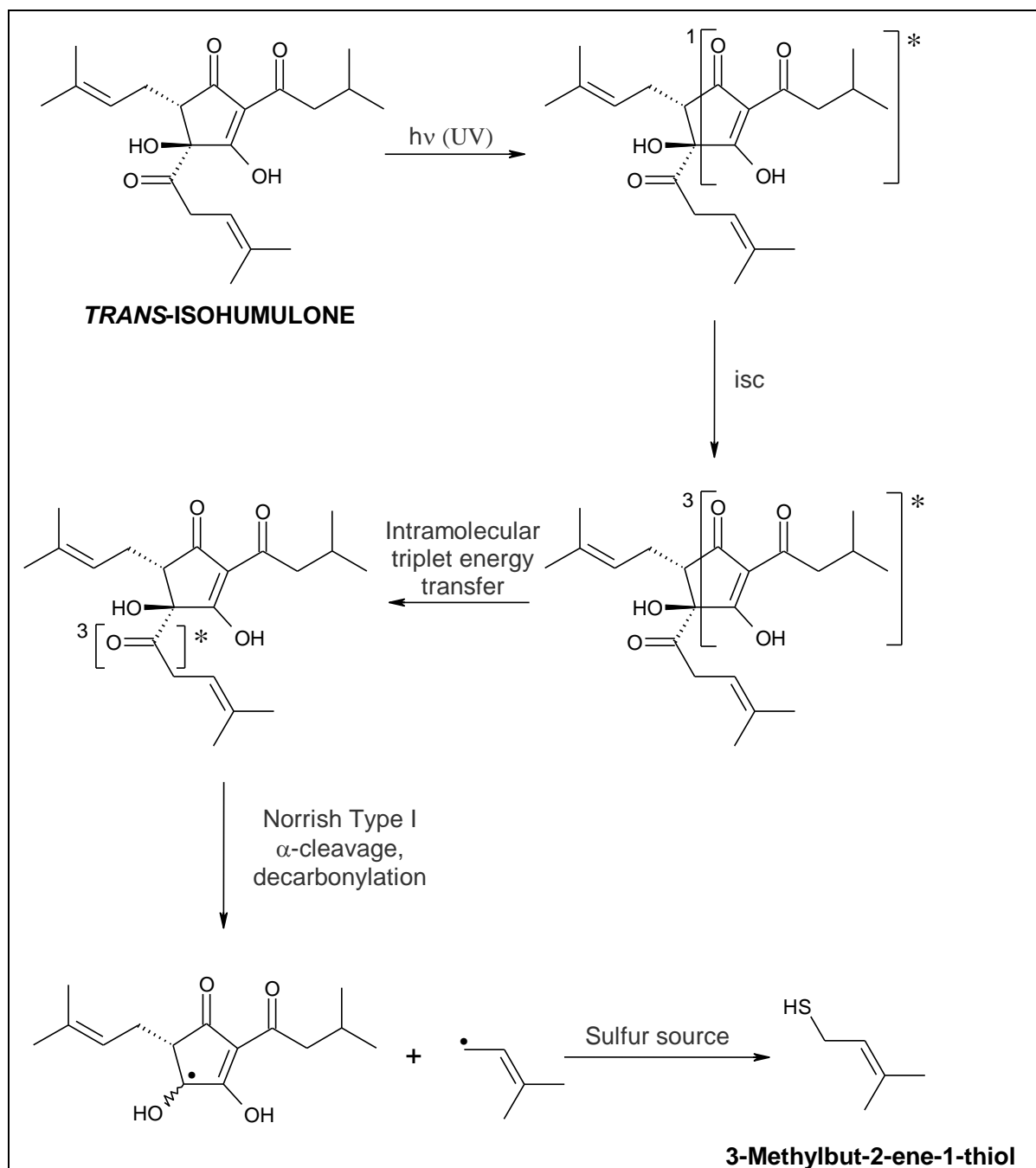
with respect to the TREPR signal derived from laser-flash photolysis of a mixture containing dihydroisohumulones and flavin mononucleotide, or of a model system consisting of EDTA and flavin mononucleotide. The superimposed signal is derived from further degradation of the cation radicals resulting from one-electron transfer of isohumulones or tetrahydroisohumulones to triplet-excited flavin mononucleotide.

Although dihydroisohumulones underwent one-electron transfer in the presence of a triplet-excited flavin, no photolysis of dihydroisohumulones was apparent, even at prolonged exposure times, suggesting occurrence of back-electron transfer. Important photoreaction products found on sensitized irradiation of isohumulones include riboflavin adducts containing a 3-methylbut-2-enyl side chain, oxidized five-membered ring fragments, and decarbonylated isohumulones. The distribution of photoreaction products for tetrahydroisohumulones is very similar, except for riboflavin adducts which were found to include addition of both 3-methylbutyl and 4-methylpentanoyl side chains. The identified photoreaction products are consistent with a reaction mechanism involving one-electron transfer from isohumulones or tetrahydroisohumulones in the ground state to triplet-excited riboflavin. Further degradation of the resulting cation radical leads to a 3-methylbut-2-enyl radical in conjunction with a five-membered ring fragment for isohumulones, or to a 3-methylbutyl or a 4-methylpentanoyl radical and a five-membered ring fragment for tetrahydroisohumulones.

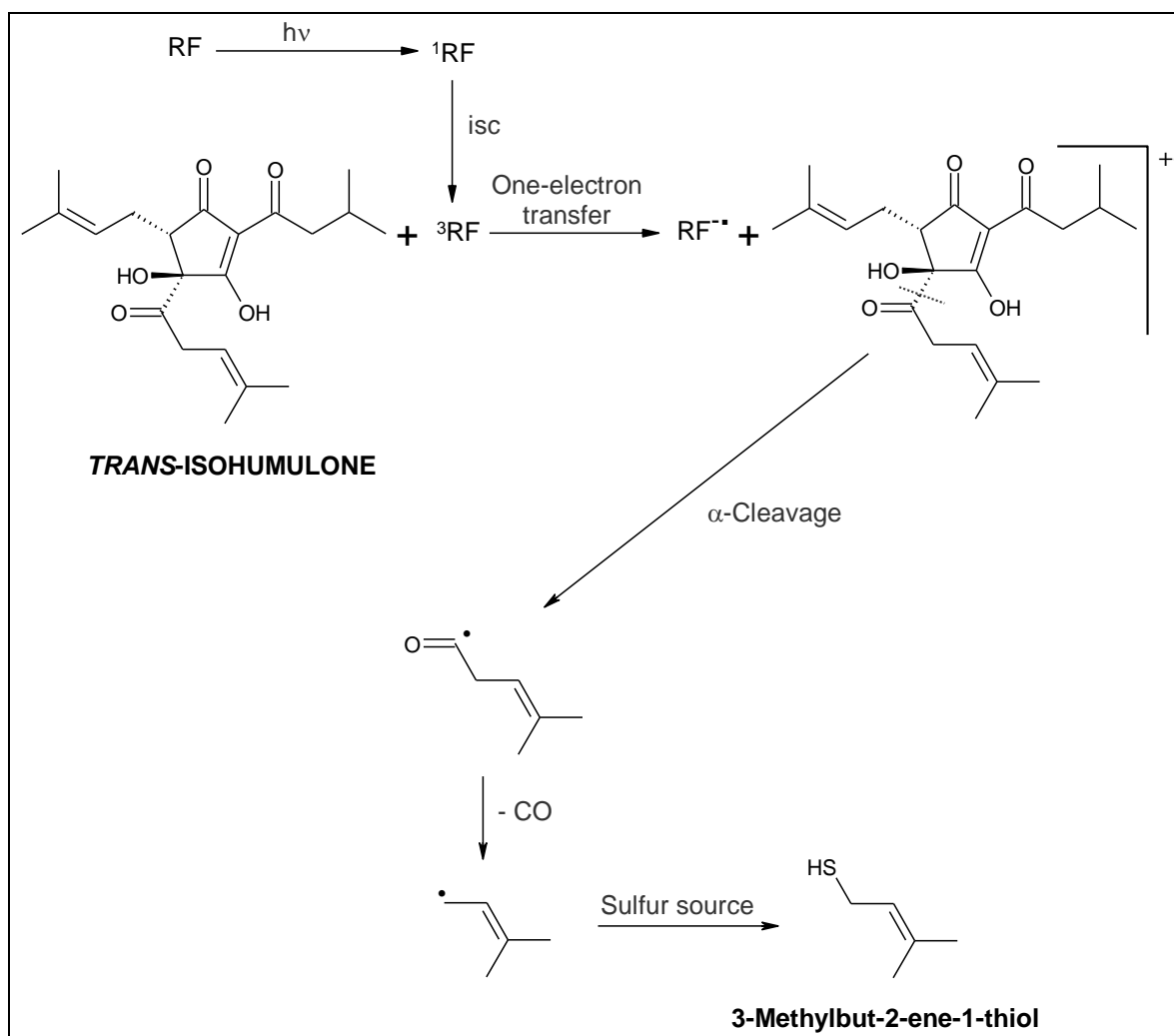
A fully innovative method to inhibit formation of the lightstruck flavor in beer results from this Ph.D. project. Filing of a patent is in preparation.

In conclusion, this Ph.D. study contributed significantly to a detailed understanding of the photolability of isohumulones and tetrahydroisohumulones with respect to the photostability of dihydroisohumulones. It was - for the first time - convincingly proven that the events leading to formation of the lightstruck flavor differ according to the irradiation conditions. On direct irradiation with UV light, energy transfer from triplet-excited isohumulones and tetrahydroisohumulones leads to Norrish Type I α -cleavage of the α -hydroxyketone chromophore resulting in formation of a 3-methylbut-2-enyl radical and a 4-methylpentanoyl radical, respectively. The 3-methylbut-2-enyl radical is the immediate precursor of 3-methylbut-2-ene-1-thiol as the key substance associated to the lightstruck flavor. Dihydroisohumulones are fully light-stable. On sensitized irradiation, triplet-excited riboflavin as a sensitizer abstracts an electron from isohumulones and tetrahydroisohumulones in the ground state thereby furnishing a cation radical, which is stabilized via cleavage of the α -hydroxyketone group representing a formal Norrish Type I α -cleavage. As a result, a 3-methylbut-2-enyl radical is formed and access to 3-methylbut-2-ene-1-thiol is imminent. Dihydroisohumulones are, again, light-stable.

Mechanisms of formation of lightstruck flavor derived from *trans*-isohumulone



Mechanism of formation of lightstruck flavor on direct irradiation
of *trans*-isohumulone with UV light.



Mechanism of formation of lightstruck flavor on sensitized irradiation of *trans*-isohumulone in the presence of riboflavin (RF) with visible light.

Samenvatting

Een onaangename geur en smaak worden geproduceerd bij het blootstellen van bier aan licht. De huidige kennis over de vorming van de lichtsmaak in bier dateert van de oorspronkelijke publicaties door Kuroiwa *et al.* in het begin van de jaren 60. Door gebruik van modelsystemen toonden zij aan dat de lichtsmaak ontstaat via een licht-geïnduceerde reactie, waarbij riboflavine als sensibilisator (riboflavine is het belangrijkste flavine in bier), isohumulonen (hop-afgeleide bitterstoffen in bier) en een zwavelbron (bijvoorbeeld cysteïne of zwavelhoudende eiwitten) betrokken zijn. De onaangename geur en smaak werden toegeschreven aan de vorming van 3-methylbut-2-een-1-thiol (MBT). Naar dit mechanisme wordt vaak verwezen in de literatuur over de lichtsmaak in bier en alle relevante gegevens, die tot op heden verzameld werden, zijn niet in tegenspraak met de Kuroiwa-premissie. Hoewel de sleutelverbindingen sinds meer dan drie decennia bekend zijn, ontbreken details over het interactiemechanisme tussen deze componenten.

Dit doctoraatsproject beoogt een grondige en gedetailleerde kennis te verwerven over het mechanisme van de vorming van lichtsmaak in bier. Deze problematiek werd benaderd via een diepgaand onderzoek van de interagerende verbindingen op moleculair niveau door modelsystemen toe te passen, bestaande uit isohumulonen, dihydro-isohumulonen of tetrahydro-isohumulonen in aan- of afwezigheid van riboflavine of flavine mononucleotide als sensibilisator.

Opheldering van een fotochemisch mechanisme vergt studies in verschillende tijdsdomeinen. Aldus werden modelsystemen onderworpen aan fotostationaire bestraling én aan fotodynamische flash-geïnduceerde experimenten. Informatie over de aard van geëxciteerde toestanden en intermediären werd, naast mechanistische kenmerken, geleverd door tijdsgeresolveerde elektron paramagnetische resonantie (TREPR) en flash-fotolyse transiënte absorptiespectroscopie (TAS). Door karakterisering en identificatie van belangrijke fotoreactieproducten via grondige structuuranalysen werden relevante reactieschema's uitgewerkt om de vorming van sleutelverbindingen te verklaren.

In een eerste fase werd een gedetailleerde studie ondernomen over de fotochemische instabiliteit van isohumulonen, dihydro-isohumulonen en tetrahydro-isohumulonen. Na laser-flash fotolyse bij 308 nm gaven isohumulonen en tetrahydro-isohumulonen intense TREPR-emissiesignalen (veroorzaakt door het triplet-mechanisme van chemisch-geïnduceerde dynamische elektronpolarisatie), terwijl, in identische omstandigheden, geen TREPR-sigitaal

waargenomen werd voor dihydro-isohumulonen. Deze bevinding levert de eerste directe spectroscopische evidentie voor de stabiliteit van dihydro-isohumulonen tegen fotolyse en bewijst éénduidig dat radicalen, geproduceerd bij fotolyse van isohumulonen en tetrahydro-isohumulonen, afkomstig zijn van de α -hydroxyketo-chromofoor, aanwezig in isohumulonen en tetrahydro-isohumulonen.

Door gefundeerde simuleringen toe te passen werden de meest voorkomende radicalen bij fotolyse van isohumulonen gekarakteriseerd als een vijftring-ketylradicaal en een 3-methylbut-2-enylradicaal, terwijl fotolyse van tetrahydro-isohumulonen leidde tot productie van een analoog vijftring-ketylradicaal en een 4-methylpentanoylradicaal. Combinatie van TREPR-gegevens liet toe een mechanisme voor te stellen voor de fotochemische afbraak van isohumulonen en tetrahydro-isohumulonen bij directe bestraling. Absorptie van UV-licht door de β -tricarbonylchromofoor leidt tot de geëxciteerde singulet-toestand. Na intersysteemkruising tot de geëxciteerde triplet-toestand, gebeurt intramoleculaire triplet-energietransfer naar het geïsoleerde α -hydroxyketon, dat, in de geëxciteerde toestand, tenslotte Norrish Type I α -splitsing ondergaat, waarbij de hoger beschreven radicalen ontstaan. Uitgebreid onderzoek van de complexe mengsels fotoreactieproducten, afgeleid van isohumulonen en tetrahydro-isohumulonen, toonde aan dat recombinitie van de belangrijkste radicalen, gekarakteriseerd via TREPR, de vorming van de voornaamste fotoreactieproducten kan verklaren, terwijl minder belangrijke fotoreactieproducten ontstaan via andere reactiewegen. De hoofdproducten bij directe bestraling van isohumulonen werden gekarakteriseerd als gedecarbonyleerde gedehydrateerde isohumulonen, terwijl de overwegende reactieproducten bij directe bestraling van tetrahydro-isohumulonen geïdentificeerd werden als dimeer-type verbindingen, gevormd door recombinitie van twee vijftring-ketylradicalen.

In een tweede fase werd de interactie tussen isohumulonen, dihydro-isohumulonen en tetrahydro-isohumulonen met riboflavine of flavine mononucleotide bij gesensibiliseerde bestraling in detail bestudeerd. Laser-flash fotolyse bij 355 nm van mengsels, bestaande uit isohumulonen, dihydro-isohumulonen of tetrahydro-isohumulonen, en riboflavine resulteerden in transiënte absorptiespectra (350-800 nm), gedomineerd door de absorptie van een neutraal flavine semichinonradicaal, terwijl de absorptie van triplet-geëxciteerd riboflavine zeer snel afnam. Deze waarnemingen zijn in overeenstemming met een één-elektrontransfer uit de grondtoestand van isohumulonen, dihydro-isohumulonen of tetrahydro-isohumulonen naar de geëxciteerde triplet-toestand van riboflavine. De één-elektrontransfer

wordt verklaard uit de kennis van de redoxpotentialen van de interagerende moleculen. De redoxpotentiaal van riboflavine verschuift van -0.3 V vs. NHE in de grondtoestand naar +1.7 V vs. NHE in de geëxciteerde triplet-toestand. Verder vertoonden TREPR-signalen bij laser-flash fotolyse van mengsels, bevattende isohumulonen of tetrahydro-isohumulonen, naast flavine mononucleotide, een overlappend signaal met het TREPR-signaal, afgeleid uit laser-flash fotolyse van een mengsel van dihydro-isohumulonen en flavine mononucleotide, of van een modelsysteem, bestaande uit EDTA en flavine mononucleotide. Het overlappend signaal wordt veroorzaakt door afbraak van de kation-radicalen, die gevormd worden door één-elektrontransfer van isohumulonen of tetrahydro-isohumulonen naar triplet-geëxciteerd flavine mononucleotide.

Hoewel dihydro-isohumulonen één-elektrontransfer ondergingen in aanwezigheid van een triplet-geëxciteerd flavine, werd geen fotolyse van dihydro-isohumulonen waargenomen, zelfs niet bij langdurige bestralingstijden, door het optreden van terug-elektrontransfer. Belangrijke fotoreactieproducten, geïdentificeerd bij gesensibiliseerde bestraling van isohumulonen, omvatten riboflavine-adducten met een 3-methylbut-2-enyl-zijketen, geoxideerde vijfringfragmenten en gedecarbonyleerde isohumulonen. De samenstelling van fotoreactieproducten uit tetrahydro-isohumulonen is zeer vergelijkbaar, behalve voor de riboflavine-adducten, die zowel 3-methylbutyl- als 4-methylpentanoyl-zijketens bevatten. De geïdentificeerde fotoreactieproducten zijn in overeenstemming met een reactiemechanisme, dat verloopt via één-elektrontransfer uit isohumulonen of tetrahydro-isohumulonen in de grondtoestand naar triplet-geëxciteerd riboflavine. Verdere afbraak van het resulterend kation-radicaal leidt tot een 3-methylbut-2-enylradicaal, samen met een vijfringfragment, voor isohumulonen, of tot een 3-methylbutyl- of een 4-methylpentanoylradicaal en een vijfringfragment voor tetrahydro-isohumulonen.

Een innovatieve methode om de vorming van de lichtsmak in bier te voorkomen werd ontwikkeld uit de resultaten van dit doctoraatsproject. Een patentaanvraag is in voorbereiding.

Samenvattend heeft dit doctoraatsonderzoek aanzienlijk bijgedragen tot een gedetailleerde kennis van de fotochemische instabiliteit van isohumulonen en tetrahydro-isohumulonen met betrekking tot de fotostabiliteit van dihydro-isohumulonen. Voor de eerste maal werd overtuigend bewezen dat de fenomenen, die leiden tot vorming van de lichtsmaak, verschillen naargelang de bestralingsomstandigheden. Bij directe bestraling met UV-licht leidt energietransfer van triplet-geëxciteerde isohumulonen en tetrahydro-isohumulonen tot een Norrish Type I fotosplitsing van de α -hydroxyketo-chromofoor, resulterend in de vorming van, respectievelijk, een 3-methylbut-2-enylradicaal en een 4-methylpentanoyl radical. Het 3-methylbut-2-enylradicaal is de directe precursor van 3-methylbut-2-een-1-thiol als de sleutelverbinding, geassocieerd aan de lichtsmaak. Dihydro-isohumulonen zijn geheel lichtstabil. Bij gesensibiliseerde bestraling met zichtbaar licht onttrekt triplet-geëxciteerd riboflavine als sensibilisator een elektron uit isohumulonen en tetrahydro-isohumulonen in de grondtoestand met vorming van een kation-radicaal, dat gestabiliseerd wordt via splitsing van de α -hydroxyketogroup volgens een formele Norrish Type I reactie. Bijgevolg ontstaat een 3-methylbut-2-enylradicaal, dat direct leidt tot 3-methylbut-2-een-1-thiol. Dihydro-isohumulonen zijn opnieuw lichtstabil.